

The role of coagulation factor XIII in the early innate immune response against streptococcal infections

Von der Fakultät für Lebenswissenschaften
der Technischen Universität Carolo-Wilhelmina
zu Braunschweig
zur Erlangung des Grades einer
Doktorin der Naturwissenschaften
(Dr. rer. nat.)
genehmigte
D i s s e r t a t i o n

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eingereicht am: 07.10.2015
mündliche Prüfung (Disputation) am: 14.12.2015

Druckjahr 2016

Vorveröffentlichungen der Dissertation

Teilergebnisse aus dieser Arbeit wurden mit Genehmigung der Fakultät für Lebenswissenschaften, vertreten durch den Mentor der Arbeit in folgenden Beiträgen vorab veröffentlicht:

Publikationen

Loof T. G., **Deicke C.**, and Medina E. (2014): The role of coagulation/fibrinolysis during *Streptococcus pyogenes* infection. *Frontiers in Cellular and Infection Microbiology* **4**: 128.

Deicke C., Chakrakodi B., Pils M. C, Dickneite G., Johansson L., Medina E., and Loof T. G. (2015): Local activation of Coagulation Factor XIII reduces systemic complications and improves the survival of mice after *Streptococcus pyogenes* M1 skin infection. *International Journal of Medical Microbiology (under revision)*.

Tagungsbeiträge

Deicke C., Rohde M., Chhatwal G.S., Nitsche-Schmitz D.P., Loof T.G. (2013): Fibrin formation as a mechanism of innate immune defense is counteracted by streptokinase. 65. Jahrestagung der Deutschen Gesellschaft für Hygiene und Mikrobiologie (DGHM), Rostock (Poster).

Deicke C., Rohde M., Dickneite G., Medina E., and Loof T.G (2014): Protective role of coagulation factor XIII in *Streptococcus pyogenes* skin infections. 66. Jahrestagung der Deutschen Gesellschaft für Hygiene und Mikrobiologie, Dresden (Poster).

*“Learning is the only thing the mind never exhausts,
never fears, and never regrets.”*

Leonardo da Vinci

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Abbreviations

%	percentage
°C	degree Celsius
μF	microfarad
μg	microgram
μl	microlitre
μm	micrometre
μM	micromol
3'	3' three prime end
5'	5' five prime end
6x his tag	6x histidin tag
A_{260}/A_{280}	absorbance at a wavelength of 260 or 280 nm
<i>ad</i>	latin 'up to'
AP	alkaline phosphatase
APS	ammonium persulfate
aPTT	activated partial thromboplastin time
APSGN	acute glomerulonephritis
ARF	acute rheumatic fever
ATP	adenosine-5'-triphosphate
bp	base pairs
BK	Bradykinin
BSA	bovine serum albumin
C	cytosine
CFU	colony forming units
cm ²	centimetre squared
CO ₂	carbon dioxide
C terminal	carboxy terminal
ctr	control
CV	column volume
C4BP	C4 binding protein
d	day
DAPI	4',6-diamidino-2-phenylindole
DC	dendritic cells
dH ₂ O	deionized water

DMSO	dimethyl sulfoxide
DNA	desoxyribonucleic acid
Dnase	deoxyribonuclease
dNTP	deoxyribonucleotide triphosphate
DTT	dithiotretol
ECL	electrochemiluminescence
ECM	extracellular matrix
<i>E. coli</i>	<i>E. coli Escherichia coli</i>
EDC	1-Ethyl-3-(3-dimethylaminopropyl)carbodiimide
EDTA	ethylenediaminetetraacetic acid
e.g.	<i>exempli gratia</i> (Latin 'for example')
ELISA	enzyme-linked immunosorbent assay
EM	electron microscopy
emm gene	gene encoding streptococcal M proteins
<i>et al.</i>	<i>et alii</i> (Latin 'and others')
F	coagulation factor
FBP	fibronectin binding protein
FCS	fetal calf serum
Fg	Fibrogammin®P
FOG	fibrinogen-binding protein of GGS
for	forward
FPA/FPB	fibrinopeptide A and B
FXIII ^{-/-}	FXIII-deficient animals
FXIIIa	activated FXIII
G	guanine
GAS	group A streptococcus
GGs	group G streptococcus
h	hour
HA	hyaluronic acid
HE	hematoxylin-eosin
HIS	histidine
HK	high molecular kininogen
hPLG	human plasminogen
IF	immunofluorescence
IFN	interferon
Ig	immunoglobulin

IL	interleukin
IMAC	immobilized metal chelate affinity chromatography
IPTG	isopropyl- β -D-thiogalactopyranoside
kb	kilobase
kDa	kilodalton
kg	kilogram
kV	kilovolt
l	litre
<i>L. lactis</i>	<i>Lactococcus lactis</i>
LB	Luria Bertani
LTA	lipoteichoic acid
M	molarity
MAC	membrane attack complex
MCS	multiple cloning site
MES	2-(N-Morpholino)ethansulfonic acid
mg	milligram
MHC	major histocompatibility complex
min	minute(s)
ml	millilitre
mm	millimetre
mM	millimol
MQ H ₂ O	milli-Q water
Mrp	M related protein
MWCO	molecular weight cut off
N terminal	amino terminal
NF	necrotizing faciitis
n. s.	non-significant
ng	nanogram
Ni-NTA	nickel nitrilotriacetic acid
nm	nanometre
N terminus	amino-terminus
Ø	diameter
O ₂	oxygen
OD ₆₀₀	optical density at 600 nm
p value	probability value
PAMP	pathogen associated molecular pattern

PBS	phosphate buffered saline
PBST	phosphate buffered saline with Tween-20
PCR	polymerase chain reaction
PFA	Paraformaldehyde
pH	<i>potentia Hydrogenii</i>
pmol	pmol picomol
PMN	polymorphonuclear cells (leukocytes)
POI	protein of interest
PRR	pattern recognition receptor
PrtI	protein I (Sfbl)
rev	reverse
RHD	rheumatic heart disease
RNA	ribonucleic acid
RNase	RNase ribonuclease
rpm	rpm rotations per minute
RT	room temperature
<i>S. pneumoniae</i>	<i>Streptococcus pneumoniae</i>
<i>S. pyogenes</i>	<i>Streptococcus pyogenes</i>
<i>S. viridans</i>	<i>Streptococcus viridans</i>
s. c.	Subcutaneous
SD	standard deviation
SDS	sodium dodecyl sulphate
SDS-PAGE	SDS-polyacrylamide gel electrophoresis
SDSE	<i>Streptococcus dysgalactiae</i> ssp. <i>equisimilis</i>
sec	second(s)
Sfbl	streptococcal fibronectin binding protein I
SIC	streptococcal inhibitor of complement-mediated lysis
SK	Streptokinase
SLO	streptolysin O
SLS	streptolysin S
SOF	serum opacity factor
SPE	streptococcal pyrogenic exotoxin
SSTI	skin and soft tissue infections
STSS	streptococcal toxic shock syndrome
T	thymine
T _A	annealing temperature

TAE	Tris-acetate-EDTA
TCR	T cell receptor
TE	Tris/EDTA
T _E	elongation time
TF	tissue thromboplastin
TNF α	tumor necrosis factor α
tPA	tissue plasminogen activator
pmol	picomol
rev	reverse
T ₆₀₀	transmission at a wavelength of 600 nm
TAFI	thrombin-activatable fibrinolysis inhibitor
TEM	transmission electron microscopy
TEMED	N,N,N,N-tetramethylethylenediamine
THB	todd Hewitt broth
THY	todd Hewitt broth with yeast extract
U	unit
uPA	urokinase-type plasminogen activator
UV	ultraviolet
V	volt
v/v	volume per volume
w/v	weight per volume
wt	wild type
α	alpha
β	beta
γ	gamma
Δ	delta
E	molar extinction coefficient
Ω	ohm

1 ABSTRACT

Although coagulation is an essential process of hemostasis and wound healing, a number of studies published over the last two decades delineate an additional role of the intrinsic pathway of coagulation, also known as “contact system”, in the early innate immune response against bacterial infections. Thus, activation of the contact system on the surface of bacteria such as *Streptococcus pyogenes* (*S. pyogenes*) leads to the induction of the entire clotting cascade resulting in pathogen immobilization within the generated fibrin network. As coagulation factor XIII (FXIII) was identified as a key factor within this process, the overall objective of this study was the further investigation of the importance of FXIII during streptococcal infections *in vitro* and *in vivo*.

Results obtained from *in vitro* clotting assays indicated that FXIII selectively influenced the entrapment of *S. pyogenes* bacteria of the serotype M1. The relevance of this FXIII-mediated entrapment mechanism for the host defense against *S. pyogenes* M1 was investigated *in vivo* using a murine model of streptococcal skin and soft tissue infection. Mice deficient in the expression of FXIII developed more severe infection and systemic complications after subcutaneous inoculation with *S. pyogenes* M1 than wild type animals as demonstrated by prolonged clotting times, elevated levels of the pro-inflammatory cytokine interleukin 6 (IL-6), increased bacterial dissemination and decreased survival. These results clearly illustrated a protective role of FXIII during *S. pyogenes* M1 infection. As a proof of principle, local reconstitution of FXIII-deficient mice with a human FXIII-concentrate (Fibrogammin®P) applied subcutaneously 1 h after *S. pyogenes* inoculation resulted in reduced systemic complications and an improved survival of infected FXIII-deficient mice.

The results in this thesis also demonstrate the capacity of the M1 protein of *S. pyogenes* to inhibit the conversion of fibrinogen to fibrin during *in vitro* clotting, a mechanism that could help *S. pyogenes* bacteria of serotype M1 to counteract their immobilization within fibrin clots. Moreover, the A region of the streptococcal M1 protein was required for the inhibition of the fibrinogen conversion. However, it could be demonstrated that FXIII counteracted the M1 protein-mediated inhibition of fibrinogen to fibrin conversion and supported the entrapment of *S. pyogenes* within the fibrin network. In the absence of FXIII, the lack of bacterial immobilization may explain the more extensive bacterial dissemination and infection severity observed in FXIII-deficient mice after subcutaneous inoculation with *S. pyogenes* M1.

Results obtained in the context of this thesis support the concept of a therapeutic potential of FXIII and might lead to the development of novel antimicrobial strategies for the treatment of skin and soft tissue infections caused by *S. pyogenes* of serotype M1.

2 INTRODUCTION

2.1 The genus *Streptococcus*

Bacteria of the genus *Streptococcus* are Gram-positive organisms with a size of 0,6 to 1 μm . The origin of the name *Streptococcus* arises from the ancient Greek words “kókkos”, which characterizes the coccoid shape of these bacteria, and “streptós” that represents the property of these bacteria to arrange in chains or pairs (see Figure 2.1). Streptococci are non-motile bacteria that do not form endospores. The genus *Streptococcus* belongs to the family of *Streptococcaceae* and to the order of *Lactobacillales*. This may explain why streptococci generate their energy mainly by fermentative conversion of carbohydrates into lactate. Streptococci are facultative anaerobic and aerotolerant bacteria that grow best in nutrient-rich media.

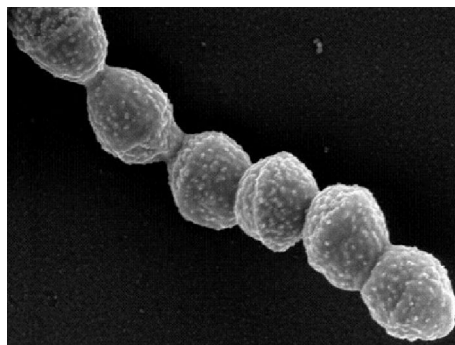


Figure 2.1: Electron microscopy image of *Streptococcus pyogenes*.

Streptococcus pyogenes is the most prominent representative of the genus *Streptococcus*. These coccoid pathogenic bacteria arrange in chains or pairs and can be coated with a hyaluronic acid capsule. This image was kindly provided by Prof. Dr. Manfred Rohde, Central Facility for Microscopy, HZI.

The genus *Streptococcus* comprises numerous different species which can be classified by several methods. One of the first described methods was the classification of streptococci by their ability to lyse erythrocytes on blood agar, termed hemolysis (Brown, 1919; Taranta and Moody, 1971). Three types of hemolysis are distinguished: α -, β - and γ -hemolysis (see Figure 2.2). Alpha-hemolytic streptococci like *Streptococcus pneumoniae* (*S. pneumoniae*) mediate the oxidation of iron in the hemoglobin molecules within the erythrocytes and thus cause a change in absorbance maximum of hemoglobin. The result is a greenish zone around the bacterial colonies within the blood agar. *Streptococcus pyogenes* (*S. pyogenes*) belongs to the β -hemolytic bacteria that completely lyse erythrocytes, resulting in clear zones around bacterial colonies growing in blood agar. Streptococci that do not lyse erythrocytes at all are called γ -hemolytic (e.g. *Streptococcus salivarius*).



Figure 2.2: Types of hemolysis.

Hemolysis can be classified into three types: the α -hemolysis mediated by *S. pneumoniae* (A), the β -hemolysis promoted by *S. pyogenes* (B) and γ -hemolysis observed for *Streptococcus salivarius* (C) (modified from <https://www.studyblue.com/notes/note/n/beta-hemolytic-strep/deck/3989611>).

The enormous antigenic variation among β -hemolytic streptococci allows their further classification into different serogroups following the scheme developed by Rebecca Lancefield in the 1930s. The Lancefield typing is based on the serological detection of antigenic differences of the C polysaccharide within the cell wall of β -hemolytic streptococci allowing their differentiation in Lancefield groups A to H and K to V (Lancefield, 1933). In the context of this thesis, most studies are focused on *S. pyogenes*, the most prominent representative of the genus *Streptococcus*. The C polysaccharide of *S. pyogenes* is composed of N-acetyl- β -D-glucosamine linked to a rhamnose polymer backbone (Zelevnick *et al.*, 1963; Cunningham, 2000). *Streptococcus dysgalactiae* ssp. *equisimilis* (SDSE), an important representative of the group G streptococci (GGS), is also a subject of this thesis.

2.2 *Streptococcus pyogenes*

S. pyogenes or group A streptococcus (GAS) is a major human pathogen worldwide with an estimated global mortality comparable to infectious diseases caused by rotavirus, measles and *Haemophilus influenzae* type B (Carapetis *et al.*, 2005). More than 1,78 million new cases of severe *S. pyogenes* infections causing more than 500 000 deaths are documented every year. Additionally, 111 million cases of streptococcal pyoderma and 616 million cases of *S. pyogenes*-mediated pharyngitis were reported annually (Carapetis *et al.*, 2005).

S. pyogenes exhibits great genetic and phenotypic variations that allow their further discrimination into serotypes by a molecular biology method called *emm* typing. This typing system is based on the DNA sequence analysis of the gene that encodes the hypervariable amino terminal (N terminal) part of the M protein (*emm* gene), an important surface protein of *S. pyogenes*. By using this method, more than 130 different *S. pyogenes* serotypes (Centers for Disease Control and Prevention, 2012) correlating with more than 220 *emm* genotypes have been differentiated up to now (Sanderson-Smith *et al.*, 2014). This enormous diversity within the *emm* gene is thought to be a result of horizontal gene transfer by intragenic and

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intergenic recombination within *S. pyogenes* species as well as with other streptococcal species like *S. dysgalactiae* and GGS (Carapetis *et al.*, 2005; Simpson *et al.*, 1992).

2.2.1 Pathogenic potential of *Streptococcus pyogenes*

S. pyogenes typically colonizes the mucosal surfaces and the skin and can be carried asymptotically as a part of the human microbiota of the skin or respiratory tract (Tart *et al.*, 2007). Asymptomatic carriers represent the main reservoir for these bacteria because *S. pyogenes* mainly gets transmitted by respiratory droplets and direct person-to-person contact (Wong and Yuen, 2012). The factors influencing the development of a *S. pyogenes* infection include environmental, genetic, acquired host factors such as previous infections and the host immune status.

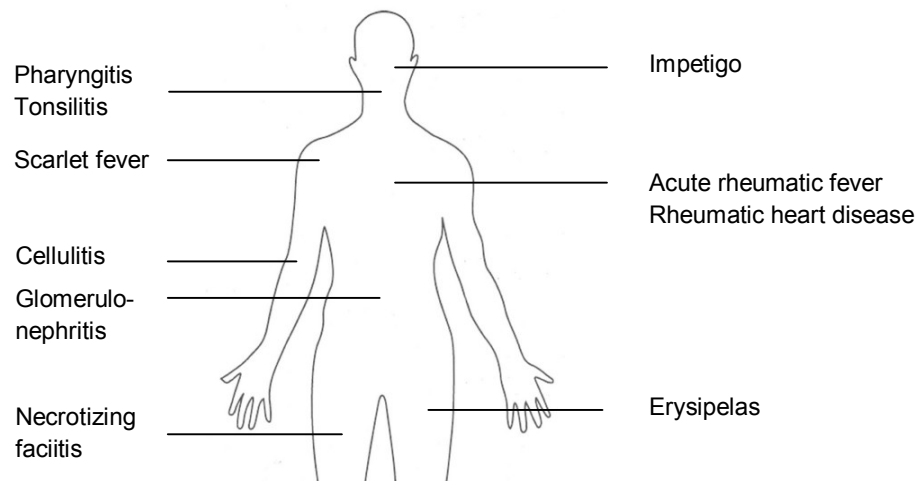


Figure 2.3: Entry and infections sites of *Streptococcus pyogenes*.

S. pyogenes causes a broad range of infectious diseases ranging from mild, often self-limiting infections to severe, life-threatening diseases. Rheumatic heart disease and glomerulonephritis are possible sequelae that can follow recurrent, non-treated *S. pyogenes* infections (modified after <http://classroom.sdmesa.edu/eschmid/lecture16-microbio.htm> and http://static.cosmiq.de/data/question/de/aae/80/aae8091052845d71ff7abe30c1cc9287_1_orig.jpg).

The ability of *S. pyogenes* to cause a broad range of different diseases represents a special feature of these bacteria (see Figure 2.3). Hence, this versatile, human specific pathogen is responsible for mild, often self-limiting infections, but also for severe systemic, life-threatening diseases. Accordingly, diseases caused by *S. pyogenes* can be divided into four classes: superficial infections, invasive infections, toxin-mediated infections and non-suppurative complications (Jaggi and Shulman, 2006).

2.2.1.1 Superficial infections

Most frequently, the origin of superficial *S. pyogenes* infections is an infection of the mucosa

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or epithelial membranes of the skin or upper respiratory tract causing pharyngitis, tonsillitis, Impetigo and pyoderma (Tart *et al.*, 2007). *S. pyogenes*-mediated pharyngitis represents about 30 % of all acute infections in children between 5 and 15 years (Bisno, 1995). Typical clinical symptoms of this disease include sudden-onset fever combined with a sore throat causing an inflammation of the pharynx and tonsils (Walker *et al.*, 2014). Pyoderma and its special variant *Impetigo* are *S. pyogenes*-mediated infections of upper layers of the skin affecting mostly the epidermis of the face and extremities (Bisno, 1996).

2.2.1.2 Invasive infections

Life-threatening, severe *S. pyogenes* infections are characterized by extensive tissue damage, vascular dissemination of bacteria and severe disease manifestation resulting in a high morbidity and mortality (Olsen *et al.*, 2009). A typical starting point of invasive streptococcal infections might be a superficial infection after injury of the skin followed by invasion of the pathogen into deeper tissue. By transgression of anatomical barriers, *S. pyogenes* is able to disseminate through the blood stream and invade deeper muscle and skin layer causing invasive diseases, such as necrotizing fasciitis (NF), bacteremia, cellulitis and sepsis.

NF is associated with very high levels of human morbidity and high mortality rate due to the rapid progress of this disease (Olsen and Musser, 2010). The key mechanisms of this highly effective invasion of the host include the coordinated expression of virulence factors, tissue damage and vascular dissemination. Additionally, *S. pyogenes* can impair the host immune response and adapt very fast to the new host environment by rapidly adjusting its transcriptional response (Olsen *et al.*, 2009). Also genetic and acquired host factors like antecedent viral infections influence NF pathogenesis (Olsen and Musser, 2010). The excessive tissue damage during NF is caused by tissue degradative enzymes produced by the invading bacteria as well as by the host immune cells such as neutrophils recruited in high numbers to the site of infection (Olsen and Musser, 2010). The main challenge for a successful treatment of NF is an early diagnosis. But as symptoms like fever, skin lesions, diarrhea, and toxemia are relative unspecific, an early diagnosis is not always feasible (Kaul *et al.*, 1997). Also the treatment of NF is very difficult, and besides intravenous antibiotic therapy consisting of penicillin, clindamycin and vancomycin, often the surgical removal of infected tissue or amputations are required (Young *et al.*, 2005). Furthermore, administration of intravenous polyclonal immunoglobulin (IVIG) was reported to have beneficial effects on the treatment of NF (Norrby-Teglund *et al.*, 2005).

2.2.1.3 Toxin-mediated infections

The streptococcal toxic shock syndrome (STSS) is an example of a toxin-mediated severe disease caused by *S. pyogenes* (Cone *et al.*, 1987). STSS can be accompanied by NF and is mediated by bacterial superantigens belonging to the family of streptococcal pyrogenic exotoxins (SPEs) (Llewelyn and Cohen, 2002; Bisno *et al.*, 2003). SPEs are potent immune-stimulators which simultaneously can bind to the major histocompatibility complex class II (MHC-II) in antigen-presenting cells and to the T cell receptor (TCR) (Marrack and Kappler, 1990). This leads to a massive activation of T cells and the concomitant excessive secretion of cytokines. The uncontrolled release of cytokines further triggers the activation of the complement system, coagulation and the fibrinolytic system that might result in hypertension and multi-organ failure, the two classical characteristics of STSS (Bisno *et al.*, 2003).

Scarlet fever is another toxin-mediated streptococcal disease that is caused by an infection of human pharynx with *S. pyogenes* strains secreting bacteriophage-encoded SPEs like SPE-A and -C (Walker *et al.*, 2014). Typical symptoms of scarlet fever include deep red, finely papular, erythematous rash, the so-called “strawberry tongue” and exudative pharyngitis (Henningham *et al.*, 2012).

2.2.1.4 Post-infection sequelae

Recurrent non-treated *S. pyogenes*-mediated superficial infections of the skin or pharynx can lead to post-infection immune sequelae like acute rheumatic fever (ARF), rheumatic heart disease (RHD) and acute glomerulonephritis (APSGN).

ARF represents the highest burden of all streptococcal sequelae, causing more than 23 300 deaths per year (Carapetis *et al.*, 2005) and is the main cause of RHD in children worldwide (Ellis *et al.*, 2010). Typical clinical presentations of ARF comprise inflammation of the joints (arthritis), the heart (carditis), the central nervous system (chorea) and the skin (erythema marginatum) (Cunningham, 2000). The diagnosis and treatment of ARF are difficult due to the absence of clear symptoms in some patients (Veasy, 2001). RHD is a chronic, autoimmune disease caused by continuous damage of heart valves mediated by repeating recrudescence of ARF. RHD and ARF are major problems in indigenous populations such as the Aboriginal Australians and underdeveloped areas with restricted access to medical care due to poverty and overcrowding (Stollerman, 1997). The pathogenesis of ARF and RHD is complex and not completely understood but is influenced by host genetic as well as environmental factors. Autoimmunity might be caused by immunological cross-reaction of antibodies against streptococcal surface structures with host proteins. This phenomenon is called “molecular mimicry” and comprises auto-antibodies which recognize, for example,

streptococcal M protein as well as host myosin, two α -helical proteins (McNamara *et al.*, 2008; Kaplan and Suchy, 1964).

Acute post-streptococcal glomerulonephritis (APSGN) is characterized by clinical symptoms such as edema, hypertension, urinary sediment abnormalities and reduced serum complement factor concentrations (Bisno, 2010). The pathogenesis is complex and includes the deposition of immune complexes, direct complement activation and cross-reactivity between antibodies against streptococcal antigens and host nephritic tissue (Friedman *et al.*, 1984; Cunningham, 2000; Henningham *et al.*, 2012).

2.2.2 Treatment and prevention of streptococcal mediated diseases

Since the 1940s, the β -lactam antibiotic penicillin has been the treatment of choice against *S. pyogenes* infections (Pichichero and Casey, 2007; Stollerman, 1993). Surprisingly, these bacteria still remain penicillin-sensitive despite intensive use of penicillin to treat these infections during the last 50 years (Macris *et al.*, 1998). Even in superficial, often self-limiting *S. pyogenes* infections, an antibiotic treatment is recommended to prevent the development of post-streptococcal immune sequelae and to reduce the infectivity and transmission to prohibit outbreaks (Bisno and Stevens, 1996). Although there is no penicillin resistance reported in *S. pyogenes*, some studies have reported failure of penicillin treatment of *S. pyogenes*-pharyngitis (Pichichero and Casey, 2007). Explanations for the penicillin tolerance of some strains include the co-existence with other β -lactamase-producing bacteria such as *Bacillus fragilis*, eradication of natural throat flora *in vivo*, carrier state, formation of biofilms as well as internalization of bacteria within epithelial host cells, a niche poorly penetrated by penicillin (Ogawa *et al.*, 2011; Brook, 2007; Pichichero and Casey, 2007). In cases of penicillin incompatibility, macrolide antibiotics like erythromycin and clarithromycin, first generation cephalosporins as well as clindamycin are effective alternative agents against *S. pyogenes* (Walker *et al.*, 2014).

Despite their exquisite antibiotic-sensitivity, a resurgence of severe invasive *S. pyogenes* infections has been observed worldwide since the 1980s. Occurrence of dominant *S. pyogenes* clones, like M1T1, through horizontal gene transfer of new toxin-encoding mobile genetic elements caused these current developments (Aziz *et al.*, 2005; Beres and Musser, 2007). The *S. pyogenes* clone M1T1, which is also subject of this study, is distributed worldwide and represents the most frequently isolated serotype in the western world (Aziz and Kotb, 2008; Cleary *et al.*, 1992). M1T1 acquired a chromosomal DNA region of 36 kb which encodes the DNase Sda1 and the superantigen SPE-A, probably responsible for the effective pathogenicity of this clone (Henningham *et al.*, 2012).

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Persistent *S. pyogenes* infections and post-streptococcal immune sequelae are severe problems, especially in geographical regions with a poor access to high standard healthcare (Henningham *et al.*, 2012). Effective control and eradication of *S. pyogenes*-mediated diseases can be only be achieved by a safe and effective vaccine. But to date, an appropriate vaccine is not available, despite of extensive research. Major difficulties in *S. pyogenes* vaccine development result from serotype diversity (McMillan *et al.*, 2013), great antigenic variation within certain serotypes (Gillen *et al.*, 2002), differences in geographical distribution of serotypes (Steer *et al.*, 2009) and the major problem of *S. pyogenes* antigens to potentially cause autoimmunity by cross-reactive antibodies recognizing human tissue structures (Kirvan *et al.*, 2003).

In the past, different *S. pyogenes* antigens including cell wall- or membrane-anchored and secreted molecules as well as group A carbohydrate were investigated for their vaccine potential (Cole *et al.*, 2008). However, vaccine research has mainly focused on the potential of the major virulence factor, the streptococcal M protein as suitable vaccine. Although *in vivo* studies using whole M protein-based vaccines showed effective protection against *S. pyogenes*-mediated pharyngitis, protection was accompanied by high auto-immunological potential (D'Alessandri *et al.*, 1978; Fox *et al.*, 1973; Polly *et al.*, 1975; Massell *et al.*, 1969). For this reason, research concentrated on the non-cross reacting amino (N) terminal, type specific part of the M protein. To date, two promising, so far safe and immunological active vaccine candidates are under investigation in different phases of clinical trials: a hexavalent and a multivalent preparation containing 6 and 26 N-terminal M protein fragments of different serotypes, respectively (Dale *et al.*, 1999; Kotloff *et al.*, 2004).

2.2.3 Virulence of *Streptococcus pyogenes*

The highly versatile pathogen *S. pyogenes* causes a broad range of clinical manifestations at various infection sites. The basis for this remarkable virulence relies in a huge repertoire of different virulence factors (see Figure 2.4).

The first step of a *S. pyogenes*-mediated infection includes the bacterial adhesion to the host tissues, which is generally mediated by streptococcal surface structures such as lipoteichoic acid (LTA), streptococcal M protein, hyaluronic acid or fibronectin binding proteins (FBP) that bind to specific receptors of the host cell (reviewed by Cunningham, 2000). In 1999, Courtney and colleagues described a two-step-model of streptococcal adhesion: the first step involves the interaction between LTA with host surface structures leading to a relatively weak adhesion; the second step involves a high-avidity adherence and tissue specificity mediated by the M protein and FBP (Courtney *et al.*, 1999; Hasty and Courtney, 1996).

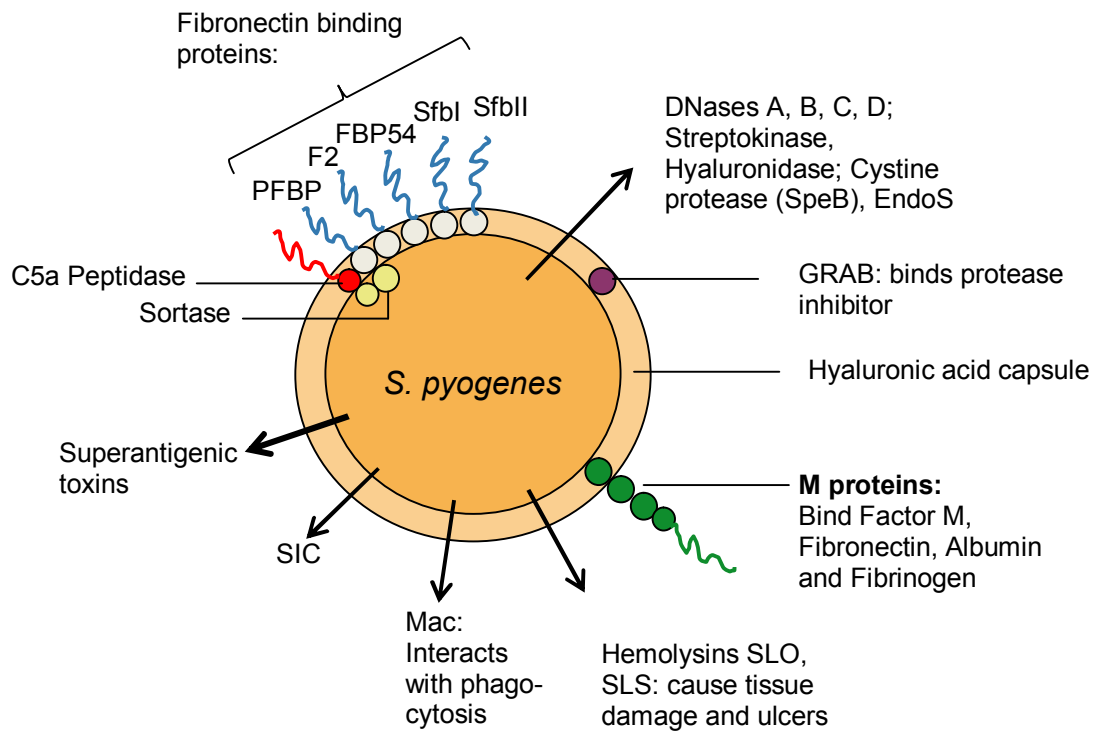


Figure 2.4: Virulence factors of *S. pyogenes*

S. pyogenes comprises a remarkable collection of surface-associated and secreted virulence factors that mediate its great virulence and capacity to induce a broad range of different diseases (modified after Mitchell, 2003).

Traditionally, *S. pyogenes* is described as a classical extracellular pathogen, however, it can internalize within human cells. The capacity of *S. pyogenes* to invade human epithelial cells was demonstrated by LaPenta and colleagues in 1994 (LaPenta *et al.*, 1994). High frequency invasion of *S. pyogenes* requires different invasins like the streptococcal M protein and/or FBPs (Fluckiger *et al.*, 1998). Internalization of *S. pyogenes* has been proposed to be an important step during its pathogenesis since it can be possibly used by the bacterium to escape the antimicrobial effect of penicillin treatment as well as the host immune defense mechanisms (Cunningham, 2000; LaPenta *et al.*, 1994).

Host colonization by *S. pyogenes* involves a rapid adaptation to the hostile environment as well as the evasion of the host immune components such as avoiding complement activation, impairing recruitment of neutrophils and inhibiting phagocytosis (Bisno *et al.*, 2003). These processes are also mediated by streptococcal virulence factors such as the M protein, the hyaluronic acid capsule as well as the streptococcal inhibitor of complement-mediated lysis (SIC).

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During the last step of infection, *S. pyogenes* tries to spread and disseminate within the host by damaging host tissues via release of streptococcal toxins like SPE-A and –C. Bacterial invasion and migration through tissue barriers is also enabled by recruitment of plasminogen via plasminogen-binding proteins such as enolase, streptokinase and glyceraldehyde dehydrogenase (Cunningham, 2000).

2.2.3.1 Surface-associated virulence factors

The surface of *S. pyogenes* is covered with a great repertoire of virulence factors that contribute to bacterial attachment, internalization and colonization of the host.

2.2.3.1.1 Streptococcal M protein

The M protein is the best studied and probably the most important virulence factor of *S. pyogenes*. The M protein contributes to attachment and invasion into host cells as well as to bacterial persistence during infection. The streptococcal M protein is a surface-associated protein that is anchored to the cell membrane by a LPXTG motif, traverses the complete bacterial cell wall and appears on the cell surface in a fibrillar, hair-like structure (Fischetti, 1991). It comprises two identical polypeptide chains that form an alpha-helical coiled-coil configuration (Hollingshead *et al.*, 1987; McNamara *et al.*, 2008). Each polypeptide chain contains four repeat regions (A, B, C and D), which differ in size due to variation in the number of certain repeats and their amino acid sequence (see Figure 2.5; Fischetti, 1989). The carboxy terminal (C terminal) part of the M protein contains the C and D repeats as well as a proline-glycine-rich and a hydrophobic membrane spanning domain. The amino acid sequence of the C and D repeats is highly conserved among *S. pyogenes* serotypes and binds factor H and human serum albumin. The central M protein domain is semi-variable and contains the B repeats, which exhibit fibrinogen-binding activity (Ringdahl *et al.*, 2000). The sequence of the extracellular N terminal domain is hypervariable and represents the basis for the serological Lancefield classification and molecular biological “emm-typing”. The resulting antigenic variation between *S. pyogenes* serotypes might be a selective advantage to avoid antibody recognition. Interaction partners of the N terminal domain are factor H and factor H like proteins, C4 binding protein (C4BP), plasminogen, immunoglobulin (Ig) A and G and fibronectin (Fischetti, 2000; Cue *et al.*, 2001).

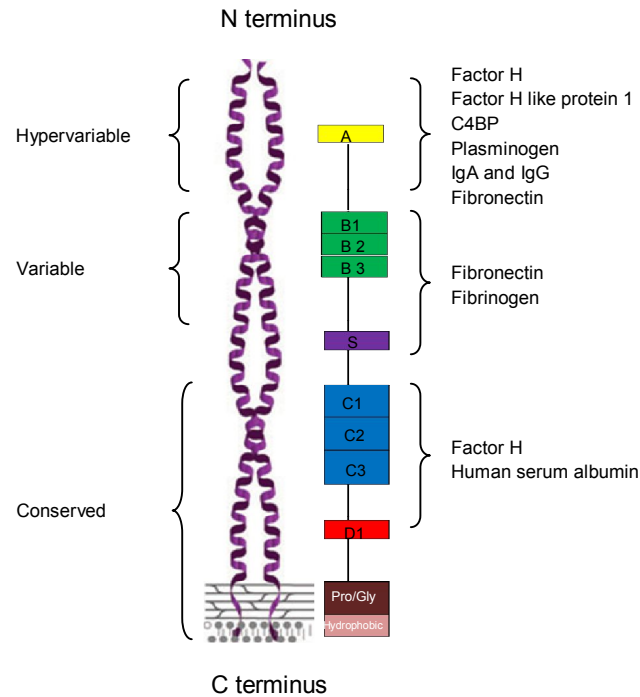


Figure 2.5: Structure of the streptococcal M1 protein.

The structure of the streptococcal M1 protein is shown schematically (right) and as structure model (left). The figure shows the M1 protein encompassing a single A repeat domain (yellow), three B repeat domains (green), a small S domain (violet), three C repeat domains (blue) and a single D repeat domain (red). M proteins are anchored to the bacterial cell membrane and traverse the whole bacterial cell wall (modified after McArthur and Walker, 2006).

The M protein was shown to mediate the bacterial adherence to HEp-2 tissue culture cells via fibronectin (Courtney *et al.*, 1986; Sanford *et al.*, 1982). The attachment of *S. pyogenes* to human epithelial and skin fibroblasts is also mediated by the M protein through the binding to cellular glucosaminoglycans or components of the extracellular matrix (ECM) (Frick *et al.*, 2003). Additionally, the streptococcal M protein is involved in biofilm formation by complex formation with LTA (Lembke *et al.*, 2006; Courtney *et al.*, 1986).

As mentioned above, *S. pyogenes* can also internalize within eukaryotic cells. One strategy for streptococcal internalization comprises the binding of fibronectin by the M protein A and B repeat regions and the interaction of fibronectin with $\alpha_5\beta_1$ -integrin that causes actin cytoskeleton rearrangements leading to bacterial uptake (Dombek *et al.*, 1999). Another internalization strategy involves the activation of intracellular signaling pathways by the M protein or M protein-fibronectin complexes (Purushothaman *et al.*, 2003). Additionally, M proteins can mediate bacterial internalization by targeting the keratinocyte surface cofactor CD46 (Rezcallah *et al.*, 2005).

The capacity of *S. pyogenes* bacteria to survive in human blood is known for a long time and could be explained by their ability to avoid phagocytosis-mediated killing by interfering with

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the human complement pathway (Oehmcke *et al.*, 2010). Phagocytosis resistance of *S. pyogenes* is mediated by several mechanisms including the inhibition of complement activation. This can be mediated by binding host serum components such as C4BP, a regulator of the classical complement pathway (Karin Berggaard *et al.*, 2001; Morfeldt *et al.*, 2001). The binding of factor H and factor H-like proteins by the streptococcal M protein prohibits the activation of the alternative complement pathway. Another strategy to resist phagocytosis consists in the binding of the plasma protein fibrinogen by the M protein. *S. pyogenes* bacteria covered with fibrinogen are not recognized by the complement factor C3-binding protein that activates the alternative complement pathway (Whitnack *et al.*, 1983; Horstmann *et al.*, 1992).

2.2.3.1.2 M like Proteins

M like proteins are Ig-binding proteins encoded by the *emm* gene superfamily (Cunningham, 2000). The overall structure of these proteins is quite similar to that of the M protein of *S. pyogenes* but differs in the types of repeat regions (Bisno *et al.*, 2003). The ability of M like proteins to bind a broad range of host proteins like IgA and IgG, albumin, fibrinogen and plasminogen as well as their contribution to phagocytosis resistance represents another similarity to *S. pyogenes* M proteins (Podbielski *et al.*, 1996; Ji *et al.*, 1998). To date, more than 20 genes belonging to *emm* gene superfamily have been identified that share more than 70 % sequence identity at their 5'ends encoding the cell-associated region of the M protein molecule (Hollingshead *et al.*, 1993). Representative proteins of *emm* like genes are for example the fibrinogen-binding protein of SDSE (FOG protein) as well as the M-related protein (Mrp), Emm and Enn (Courtney *et al.*, 2006).

2.2.3.1.3 Hyaluronic acid capsule

Many *S. pyogenes* isolates are covered with a capsule containing the high molecular weight polymer, hyaluronic acid (HA). HA consists of repeating residues of glucuronic acid and N-acetylglucosamin (Stoolmiller and Dorfman, 1969). HA synthesis is regulated by an operon containing three genes, *hasA*, *hasB* and *hasC*, representing the gene sequences for hyaluronate synthase, UDP-glucose dehydrogenase and UDP-glucose pyrophosphorylase, respectively (Crater *et al.*, 1995; Dougherty and Van de Rijn, 1992). The degree of encapsulation varies among different *S. pyogenes* serotypes and is directly connected to their phagocytosis resistance capacity (Wessels and Bronze, 1994). Thus, acapsular isogenic mutant strains show a 100-fold decrease in bacterial virulence (Wessels *et al.*, 1991). In phagocytosis resistance, HA capsule represents a physical barrier to prevent the recognition of opsonic complement proteins on the bacterial surface by phagocytes

(Cunningham, 2000). By targeting CD44 molecules on the surface of pharyngeal epithelial cells, the HA capsule also functions as an effective adhesin (Schrager *et al.*, 1998).

2.2.3.1.4 Lipoteichoic acid

Lipoteichoic acid (LTA) is described as a first step adhesin targeting fibronectin and epithelial cells. Hydrophobic interactions allow close contact between the pathogen and host cells which represent the basis of high affinity binding in the second step of adhesion (Hasty *et al.*, 1992). LTA is an amphiphatic molecule which accounts for approximately 60% of adhesion in epithelial cells (Hasty and Courtney, 1996).

2.2.3.1.5 Fibronectin binding proteins

A common attachment and invasion strategy used by *S. pyogenes* represents the binding of ECM proteins like fibronectin, collagen and laminin by streptococcal virulence factors (Walker *et al.*, 2014). Thus, fibronectin-binding proteins (FBPs) are important for the adherence to host cells in throat and skin. Fibronectin is a dimeric glycoprotein which contains a RGD (arginin-glycin-aspartate) motif that is recognized by host cell surface $\alpha_5\beta_1$ integrin (Singh *et al.*, 2010). Therefore, streptococcal FBPs function as “bridging” molecules between the bacteria and host cell surfaces. The binding of $\alpha_5\beta_1$ integrin activates intracellular signaling pathways that cause the rearrangement of actin cytoskeleton and allows the bacterial uptake (Walker *et al.*, 2014).

Streptococcal fibronectin-binding protein I (Sfbl/PrtI) (Talay *et al.*, 1992; Hanski and Caparon, 1992), SfblII/Serum opacity factor (SOF) (Kreikemeyer *et al.*, 1995), FBP 54 (Hasty and Courtney, 1996) and streptococcal M proteins belong to the most important examples of streptococcal FBPs (Bisno *et al.*, 2003). Sfbl is the most prominent FBP and plays an important role in adhesion and internalization into non-phagocytic cells like respiratory epithelial cells and cutaneous Langerhans cells (Hanski *et al.*, 1992; Okada *et al.*, 1995; Guzmán *et al.*, 1999). Expression of Sfbl is environmentally regulated by oxygen (O₂) and carbon dioxide (CO₂).

2.2.3.1.6 C5a peptidase

Another cell wall anchored streptococcal virulence factor is the proteolytic enzyme C5a peptidase. This 130-kDa serine protease is responsible for the cleavage of C5a, a complement derived chemotaxin, at its binding site for PMNs. This interaction leads to an inhibition of PMN recruitment to the site of infection and thus reduced clearance of the invading pathogen (Cleary *et al.*, 1992; Ji *et al.*, 1996; Wexler *et al.*, 1985).

2.2.3.2 Secreted virulence factors

In addition to the surface-associated virulence factors, *S. pyogenes* bacteria secrete numerous extracellular products that contribute to their pathogenesis.

2.2.3.2.1 Haemolysins

Streptolysin O (SLO) is a haemolysin produced by *S. pyogenes* strains that belongs to the family of cholesterol dependent cytolysins. SLO possesses lytic activity against all cells that contain cholesterol in their cytoplasmic membrane like PMNs, platelets and macrophages. It is irreversibly inhibited by cholesterol and O₂-labile (Mitchell, 2003; Bisno *et al.*, 2003).

Streptolysin S (SLS) is another haemolysin produced by *S. pyogenes*, especially during growth in the presence of serum and molecules like albumin and α -lipoprotein. SLS can be intracellular, cell surface bound or extracellular and is one of the most potent cytolysins which is delivered to target cells by direct contact with the bacterium (Bisno *et al.*, 2003; Ofek *et al.*, 1990). SLS is thermolabile but not influenced by O₂.

SLO and SLS are important virulence factors during the pathogenesis of NF by effectively damaging cell membranes of PMNs, platelets and subcellular organelles. Often both types of haemolysins are produced by the same *S. pyogenes* isolates (Bisno *et al.*, 2003).

2.2.3.2.2 Streptococcal pyogenic exotoxins

Streptococcal pyogenic exotoxins (SPEs) represent a family of proteins which lead to pyogenicity and have superantigenic effects on the immune system (Alouf, 1980). The special feature of these superantigens is their ability to activate much higher amounts of T cells than conventional antigens (Kotzin *et al.*, 1993; Kappler *et al.*, 1989). This effect is mediated by their property to simultaneously bind T cell receptors and MHC II molecules on antigen presenting cells that results in the stimulation of T cell proliferation and the concomitant release of high amounts of inflammatory cytokines such as tumor necrosis factor α (TNF α), interleukin (IL) 1 β and IL-2 (Fast *et al.*, 1989; Herman *et al.*, 1991; Kotb, 1995).

2.2.3.2.3 SPE-B

SPE-B, also called streptococcal proteinase, was long thought to be a streptococcal superantigen, but meanwhile it has been demonstrated that SPE-B solely exert proteolytic activity. SPE-B is a very potent cysteine protease produced by almost all *S. pyogenes* strains but in different amounts (Kapur *et al.*, 1993; Musser *et al.*, 1993). SPE-B is an important virulence factor of *S. pyogenes* during invasive diseases like NF contributing to tissue damage by cleavage of human Ig, vitronectin and other host proteins and generation of

biologically active molecules like IL-1 β , kinins and histamine (Collin and Olsén, 2001; Kapur *et al.*, 1993).

2.2.3.2.4 Streptokinase

The fibrinolytic activity of *S. pyogenes* was already observed in 1933 (Tillett and Garner, 1933) and the responsible bacterial factor was later named Streptokinase (SK) (Green, 1948). The property of *S. pyogenes* to dissolve a fibrin network represents a logical strategy to avoid the early human immune response that leads to fibrin deposition to confine the bacteria. By fibrin deposition, the infection site is targeted for further immune responses (Levi, 2004). SK is a 46 kDa protein consisting of three similar domains (α , β , γ) that are separated by two loops (Hoffmeister *et al.*, 1998). The main function of SK is the activation of human plasminogen. But, in contrast to human plasminogen, activators like urokinase or tissue plasminogen activator (uPA and tPA), SK has no intrinsic enzymatic activity. The SK proteolytic activity is generated by complex formation with human plasminogen (Reddy and Markus, 1972). Active plasminogen-SK complexes convert other plasminogen molecules to plasmin, a serine protease that is a major component of the human fibrinolytic system (Lottenberg *et al.*, 1994). In this process, SK serves as a “communication tool” between the bacteria and the host for establishing a proteolytic environment that supports the virulence and invasiveness of *S. pyogenes* (Cook *et al.*, 2012).

2.2.3.2.5 Streptococcal inhibitor of complement-mediated lysis

Another virulence factor of *S. pyogenes* that was identified in the AP1 strain of serotype M1 represents the streptococcal inhibitor of complement-mediated lysis (SIC) (Akesson *et al.*, 1996). SIC interferes with the complement-mediated lysis of erythrocytes by binding regulatory complement proteins such as clusterin and histidin-rich glycoprotein that interact with the membrane attack complex (Akesson *et al.*, 1996; Fernie-King *et al.*, 2001). The role of SIC in streptococcal virulence was demonstrated by impaired capacity of isogenic mutant strains lacking the SIC protein to colonize mice in comparison to wild type bacteria (Lukomski *et al.*, 2000).

2.3 The human coagulation system

2.3.1 Coagulation during hemostasis

Coagulation is a part of blood hemostasis, a vital process to prevent the loss of life-threatening amounts of blood after injury of blood vessels. Therefore, hemostasis is one of the fastest tissue repair systems, which is activated within seconds after vascular injury and a necessary condition for wound healing (Versteeg *et al.*, 2013). The primary step of cellular

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hemostasis comprises platelet aggregation at the site of injury and the formation of a loose platelet plug, while the coagulation process per se represents the secondary, or plasmatic, hemostasis which ensures plug stabilization by formation of a stable fibrin network. Beside, preventing the excessive loss of blood cells and plasma proteins, rapid wound healing also avoids the entrance of pathogenic microorganisms (van der Poll and Herwald, 2014).

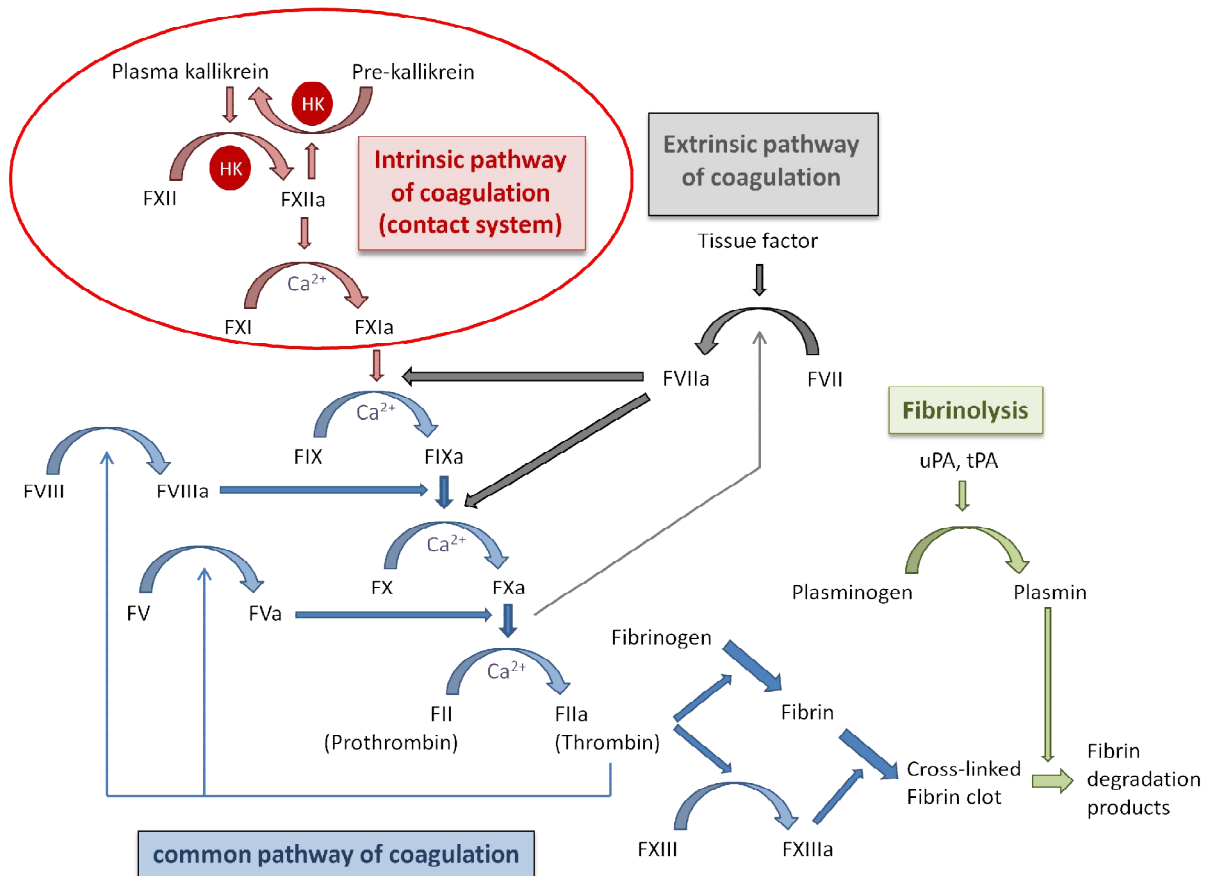


Figure 2.6: Schematic representation of coagulation cascade and fibrinolysis.

The blood coagulation is initiated by an extrinsic (grey) and an intrinsic (red) pathway of coagulation which both finally lead to the activation of the common pathway of coagulation (blue). To ensure hemostasis, the fibrinolytic system (green) mediates the degradation of fibrin clots. Coagulation factors are indicated with “F”, the addition “a” represents the active form, uPA/tPA: urokinase/tissue plasminogen activator; HK: high molecular weight kininogen (Loof, Deicke and Medina, 2014).

The blood coagulation cascade is initiated by an intrinsic or an extrinsic pathway, both leading to the activation of the common pathway (see Figure 2.6). The common pathway is initiated by the activation of factor X (FX) which further activates prothrombin to thrombin. Thrombin then catalyzes the conversion of fibrinogen to fibrin which subsequently forms a fibrin network by the clustering of fibrin monomers into fibrils. Afterwards, fibrin fibrils are stabilized by thrombin-activated factor XIII (FXIII). During thrombin activation, early

generated thrombin induces the feedback activation of factor V (FV) and factor VIII (FVIII) (Brummel *et al.*, 2002).

2.3.1.1 Extrinsic coagulation pathway

Tissue factor or tissue thromboplastin (TF) is the key activator of the extrinsic coagulation pathway (see Figure 2.6). This membrane-spanning glycoprotein is constitutively expressed on many extravascular cells like blood vessel surrounding fibroblasts, pericytes and epithelial cells (Breitenstein *et al.*, 2010; Wilcox *et al.*, 1989). Besides its main function in activation of coagulation, TF also mediates several inflammatory reactions by inducing the release of cytokines, chemokines and adhesion factors (Åberg and Siegbahn, 2013; Chu, 2005; Srinivasan and Bogdanov, 2012). After injury and damage of vascular endothelial cells, TF is exposed to the blood plasma and forms a catalytic complex with factor VII (FVII), which further activates factor IX (FIX) and FX (see Figure 2.6; Delvaeye and Conway, 2009). FX leads to the activation of thrombin on the platelet surface and the induction of the common coagulation pathway that finally promotes further coagulation and thrombus formation.

2.3.1.2 Intrinsic coagulation pathway

The intrinsic pathway of coagulation, also known as the contact system, includes four factors: the proteases FXII, FXI and plasminogen (PK) as well as the non-enzymatic co-factor high molecular kininogen (HK) (see Figure 2.6; Frick *et al.*, 2007). Under physiological conditions, these factors circulate as zymogens in the blood or are assembled to endothelial cells, platelets or PMNs (Colman and Schmaier, 1997). Their activation is induced by endothelium changes from anti- to pro-coagulant status e.g. after injury. Contact factors such as FXII assemble on negatively charged surfaces like kaolin, glass, dextran sulfate, biological material like DNA, RNA and collagen as well as the surfaces of gram-positive and gram-negative bacteria (Maas *et al.*, 2011; Ben Nasr *et al.*, 1997; Herwald *et al.*, 1998; Srisakandan *et al.*, 2000). By binding to a bacterial surface, FXII is autocatalytically activated into FXIIa which in turn activates PK and FXI, both anchored to the bacterial surface by HK (see Figure 2.6; Ben Nasr *et al.*, 1995). FXI triggers the common pathway of coagulation by activating FIX and FX whereas PK cleaves HK which leads to the generation of the proinflammatory and vasoactive peptide bradykinin (BK) (Joseph and Kaplan, 2005; Bhoola *et al.*, 1992).

2.3.1.3 Fibrinogen and Fibrin

The formation of a stable fibrin clot is the critical step of the coagulation process that ensures wound closure and prevents the invasion of pathogens. Monomeric fibrinogen is a soluble precursor protein consisting of two α -, two β - and two γ -polypeptide chains building up a

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complex fibrous heterohexamer (α A, β B, γ_2) with a total molecular weight of 340 kDa (Weisel, 2005; Doolittle, 1983). The six polypeptide chains are intramolecularly linked by 29 disulfide bonds (Henschen and McDonagh, 1986). The complex fibrinogen molecule contains a central, globular E domain, built by the N terminal ends of the α -, β -, and γ -chain, as well as two globular D domains (see Figure 2.7). The fibrinogen D domains are located at both ends of a fibrinogen molecule and contain the C terminal ends of the α -, β -, and γ -subunits (see Figure 2.7). The external globular D domains are connected with the central E domain by triple-stranded α -helical coiled-coiled rods (reviewed by Weisel, 2005).

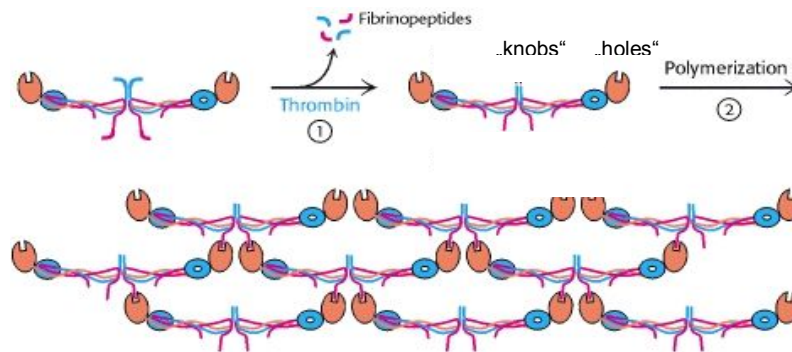


Figure 2.7: Formation of a fibrin clot during coagulation.

Fibrinogen consists of two α (orange), two β (blue) and two γ polypeptide chains (pink) building up two external globular D domains and a central globular E domain connected by α -helical coiled-coiled rods. The conversion of fibrinogen to fibrin is catalyzed by thrombin and induces the cleavage of fibrinopeptides (1). Fibrin polymerization is mediated by interactions between C terminal "knobs" and N terminal "holes" of the β - and γ -subunits (2) (Freeman, 2012).

During clot formation, soluble monomeric fibrinogen molecules are converted into fibrin by the thrombin-mediated enzymatic cleavage of four arginin-glycine bonds within the E domain releasing the fibrinopeptides A and B (FPA and FPB) from the N termini of both α - and β -subunits of fibrinogen (see Figure 2.7; Blombäck *et al.*, 1978). Although FPA and FPB constitute less than 2% of the total molecular weight, the cleavage has profound structural consequences that allow fibrin polymerization (Yang *et al.*, 2000). The newly formed N termini of the α - and β -chain within the central E domain expose new binding sites called "knobs". Corresponding "holes" are exposed by β - and γ -chains C termini. The binding of "knobs" and "holes" allows the spontaneous assembly of fibrin molecules into protofibrils, which further aggregate three-dimensional fibers. This process is mediated by intermolecular interactions between different fibrin molecules (reviewed by Mosesson, 2005; Weisel, 2005). During fibrin polymerization, γ -dimers are generated by assembly of two γ -chains of different fibrin molecules. Furthermore α -polymers are generated by association of several α chains (Mosesson, 2005; Francis and Marder, 1987).

In addition to the important function of fibrinogen/fibrin during coagulation, these molecules exert additional functions in wound healing, inflammation and angiogenesis by interaction with a broad range of molecules like fibronectin, albumin, thromboplastin, von Willebrand factor, fibroblasts and vascular endothelial growth factor (reviewed by Weisel, 2005).

2.3.1.4 Fibrinolysis

To prevent blood loss and ensure blood fluidity, hemostasis is a tightly regulated process (Kolev and Machovich, 2003). Fibrinolysis represents one part of these regulation processes that is responsible for the sequential degradation of fibrin clots into distinct degradation products (Cesarman-Maus and Hajjar, 2005; Walker and Nesheim, 1999). Fibrinolysis and coagulation are simultaneously activated whereas fibrinolysis operates more slowly. The major fibrinolytic protease plasmin is generated from the zymogen plasma protein plasminogen by tPA and uPA as well as by FXIIa and PK, linking the contact system to fibrinolysis (see Figure 2.6; Maas *et al.*, 2011). The fibrinolytic system is regulated by tPA and uPA as well as by plasminogen activation inhibitors like PAI-1, α -antiplasmin or TAFI (thrombin-activatable fibrinolysis inhibitor) (reviewed by Longstaff and Kolev, 2015 and van der Poll and Herwald, 2014).

2.3.2 Coagulation as a part of innate immunity against streptococcal infections

The hemostasis, including coagulation, and the host innate immune system have been viewed as two entirely separated systems in the past. However, during the last two decades a growing body of evidence has been provided demonstrating that coagulation also participates in the early host innate immune defense against pathogens. It was demonstrated that coagulation plays an active role in the containment and elimination of invading pathogens, including *S. pyogenes* (Loof *et al.*, 2010; Esmon *et al.*, 2011; Loof *et al.*, 2011a; Wang *et al.*, 2010). It was also shown that coagulation activates several innate immune mechanisms including the release of antimicrobial peptides (AMPs) from platelets, generation of AMP during clotting, induction of the cellular immune response by recruitment, attachment and activation of phagocytes as well as the activation of pro- and anti-inflammatory reactions (Frick *et al.*, 2006; Kirschenbaum *et al.*, 2004; Shpacovitch *et al.*, 2008).

2.3.2.1 Innate immune response against *Streptococcus pyogenes*

The fundamental basis of an effective defense against invading pathogens includes the cooperation of various chemical, physical, enzymatic and cellular components of the immune system. Traditionally, the mammalian immune system is classified into the rapid, but rather

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unspecific, innate immune response and the highly specialized, but more slowly acting, adaptive immune response. Whereas current literature provides a general overview about the host innate immune mechanisms against *S. pyogenes* infections, the role of the adaptive immune response including highly specialized T and B lymphocytes as well as highly specific antibodies is less well studied (Fieber and Kovarik, 2014).

The initiation of the host immune response to fight *S. pyogenes* requires the recognition of the invading bacteria through pathogen-associated molecular patterns (PAMPs), which are conserved molecules or structures within pathogens that are not present in the host. The innate immune system is activated upon recognition of PAMPs by the corresponding host pathogen recognition receptors (PRRs) such as Toll-like receptors (TLRs). Recognition leads to the induction of intracellular signaling pathways leading to the activation of transcription factors such as NF κ B and the production of chemokines and cytokines involved in the recruitment of immune cells (see Figure 2.8) (Medzhitov *et al.*, 1998; Kawai and Akira, 2010). Several studies using murine *S. pyogenes* infection models identified the signaling adaptor MyD88 as a key component of the initiation of the immune defense and inflammatory response against *S. pyogenes*-mediated infections (Loof *et al.*, 2010, 2008).

The innate immune response against *S. pyogenes* is complex and includes the recruitment and activation of PMNs, macrophages and dendritic cells (DCs) (Fieber and Kovarik, 2014; Goldmann *et al.*, 2004; Loof *et al.*, 2007; Mishalian *et al.*, 2011). The complete repertoire of innate immune components and their relevance are not completely understood today. Nevertheless, studies using murine *S. pyogenes* infection models indicate the requirement of macrophages to limit bacterial spreading within the host and to ensure the survival of infected mice (Goldmann *et al.*, 2004). Macrophages are innate immune cells which are important for pathogen recognition and elimination by phagocytosis, for the induction of a local inflammatory response as well as for activation of the adaptive immunity by antigen presentation on MHC II molecules (Cavaillon and Adib-Conquy, 2005). Macrophages are the major producer of TNF α and simultaneously are highly responsive to this cytokine (Parameswaran and Patial, 2010). TNF α is also secreted by DCs (Loof *et al.*, 2008), which are highly specialized antigen-presenting cells that mainly contribute to antigen capture and processing as well as the stimulation of other immune functions by secretion of cytokines. Other essential innate immunity components against *S. pyogenes*-mediated infections are PMNs which migrate from the blood stream to infected tissues after infection stimuli. PMNs mainly control bacterial infections by degranulation, phagocytosis, generation of massive amounts of reactive oxygen and nitrogen species, the release of inflammatory mediators like AMP, hydrolytic and proteolytic enzymes as well as limiting the dissemination of invading

pathogens by neutrophil extracellular traps (NETs) (Amulic *et al.*, 2012; Borregaard *et al.*, 2007; Brinkmann *et al.*, 2004).

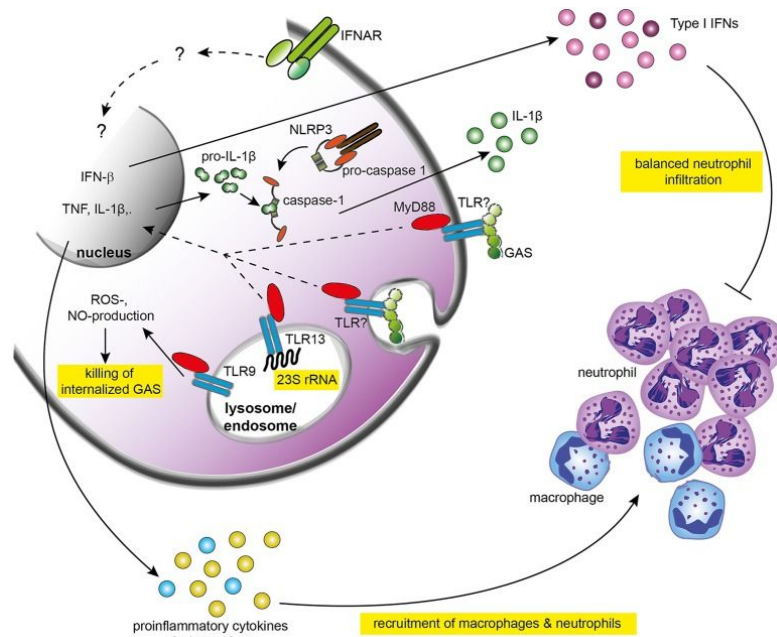


Figure 2.8: Mechanisms of *S. pyogenes* recognition and activation of the innate immune response.

Invading *S. pyogenes* bacteria are recognized by TLRs which activate intracellular signaling via the MyD88 adaptor molecule. This leads to the generation of TNF, IFN-β and IL-1β in the nucleus and to the secretion of other pro-inflammatory cytokines and chemokines recruiting macrophages and neutrophils. TLR13 is involved in the recognition of streptococcal 23s rRNA. Lysosomal TLR9 mediates killing of GAS by generating reactive oxygen and nitrogen species. Type I IFN regulates infiltration of neutrophils by a process which is only poorly understood, to date (Fieber and Kovarik, 2014).

2.3.2.2 Bacterial entrapment within fibrin networks

In addition to previously described pro-inflammatory innate immune functions (see section 1.2.2), coagulation is also involved in the containment and elimination of *S. pyogenes* (Loof *et al.*, 2011b). Activation of the intrinsic pathway of coagulation by *S. pyogenes* is mediated by the binding and activation of FXII on the bacterial surface that was already described in section 2.2.1.2. Beside FXII, also FV, FXI, HK and fibrinogen assemble on *S. pyogenes* surface by interactions with bacterial surface proteins such as M proteins (Herwald *et al.*, 2003b). The assembly of contact factors leads to the activation of the entire clotting cascade including the activation of prothrombin, fibrinogen and FXIII and leading to the formation of a fibrin clot (Loof *et al.*, 2011b). During clotting, *S. pyogenes* is entrapped and immobilized within the fibrin network where bacteria are confined and their dissemination and spreading within the host is prevented (see Figure 2.9; Loof *et al.*, 2011b; Sun *et al.*, 2004; Sun, 2006; Matsuda *et al.*, 2007). In addition to the confinement of bacteria at the site of infection, the

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coagulation system also exerts bactericidal activities. Several AMPs are generated during coagulation including fragments of the D3 and D5 domain of HK, host defense peptides generated from thrombin as well as fibrinogen-derived AMPs (Frick *et al.*, 2006; Nordahl *et al.*, 2005; Papareddy *et al.*, 2010; Pålman *et al.*, 2013). Additionally, Loof and colleagues reported cell membrane disruption and bacterial killing within fibrin clots (Loof *et al.*, 2011b).

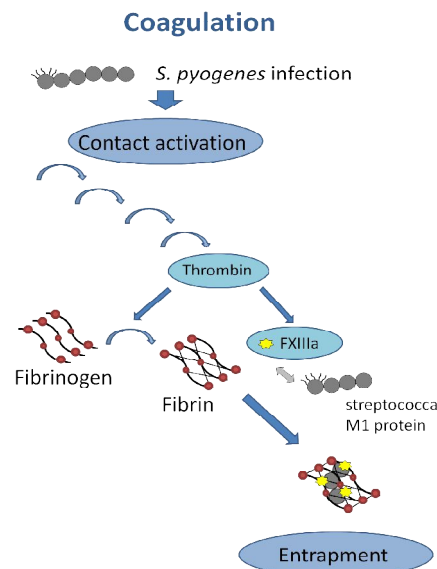


Figure 2.9: Overview of the entrapment of *S. pyogenes* M1 from fibrin clots.

After *S. pyogenes* infection, the host contact system is activated on the bacterial surface leading to the induction of the entire clotting cascade and subsequent FXIII-mediated entrapment of *S. pyogenes* within the fibrin clot. Immobilized bacteria are not able to disseminate within the host and the systemic course of infection is prevented (Loof, Deicke and Medina, 2014).

The protective role of coagulation in innate immunity is highly conserved among invertebrates and vertebrates (Loof *et al.*, 2011a). *Drosophila* transglutaminase (TG) and mammalian FXIII, respectively, represent the only clotting factors that have been conserved during evolution (Theopold *et al.*, 2002), suggesting an important role of TG/FXIII. A protective role of *Drosophila* TG during innate immunity was demonstrated by Wang and colleagues using a *Drosophila* infection model challenged with different bacterial pathogens (Wang *et al.*, 2010). They clearly showed that reduced TG levels resulted in increased mortality of infected larvae. TG/FXIII activity from *Drosophila* hemolymph and human plasma accumulated on microbial surface and promoted their sequestration within the clot. The activity of FXIII-dependent immobilization of *S. pyogenes* within fibrin networks as a host immune mechanism was also described for vertebrates (Loof *et al.*, 2011b). The pathogen entrapment might be promoted by interactions between FXIII and bacterial surface proteins such as streptococcal M protein. The authors demonstrated that the entrapment of *S. pyogenes in vitro* was reduced in human FXIII-deficient plasma (Loof *et al.*, 2011b).

Additionally, mice lacking FXIII develop more severe signs of inflammation after *S. pyogenes* infection when compared to wild type mice, characterized by massive neutrophil recruitment to the infection site and tissue destruction (Loof *et al.*, 2011b). The cross-linking activity of FXIII to sequester bacteria within fibrin clots was also reported for *E. coli* and *S. aureus* (Wang *et al.*, 2010).

In contrast to this positive contribution of a local induction of coagulation to the innate immunity against *S. pyogenes* infections, activation of systemic coagulation has been associated with the development of sepsis and septic shock. Systemic contact activation causes widespread fibrin deposition promoting micro- and macro-thrombosis as well as the release of BK, a pro-inflammatory and vasoactive peptide and the clinical outcome can be dramatically including multi-organ failure, reduced blood pressure, increased vascular permeability, fever and pain (Herwald *et al.*, 2003).

2.3.2.1 Importance of coagulation factor XIII

The cross-linking of fibrin polymers to generate a stable clot represents the last step of the coagulation cascade and is catalyzed by FXIII, also termed fibrin-stabilizing factor. FXIII circulates in the blood as a 320-kDa heterotetramer containing two catalytic A and two non-catalytic carrier B subunits (see Figure 2.10; Hsieh and Nugent, 2008; Yee *et al.*, 1994). The catalytic A subunits contain components responsible for the TG activity, activation peptides and substrate recognition domains, whereas FXIII B subunits seem to be responsible for the protection of the A subunits against proteolysis (Yee *et al.*, 1994). Thrombin activates FXIII by proteolysis in the presence of Ca^{2+} and fibrin. Structural changes lead to the dissociation of the B subunits and the catalytic cysteine residue of the A subunit becomes available (reviewed by Muszbek *et al.*, 2011). FXIIIa functions as a transglutaminase by catalyzing the formation of an “isopeptide” bond between a γ -glutamyl residue of a fibrin molecule and a ϵ -lysyl residue of another fibrin molecule (see Figure 2.10). In this way, fibrin polymers are stabilized by covalently cross-linking the α - and γ -chains of different fibrin molecules.

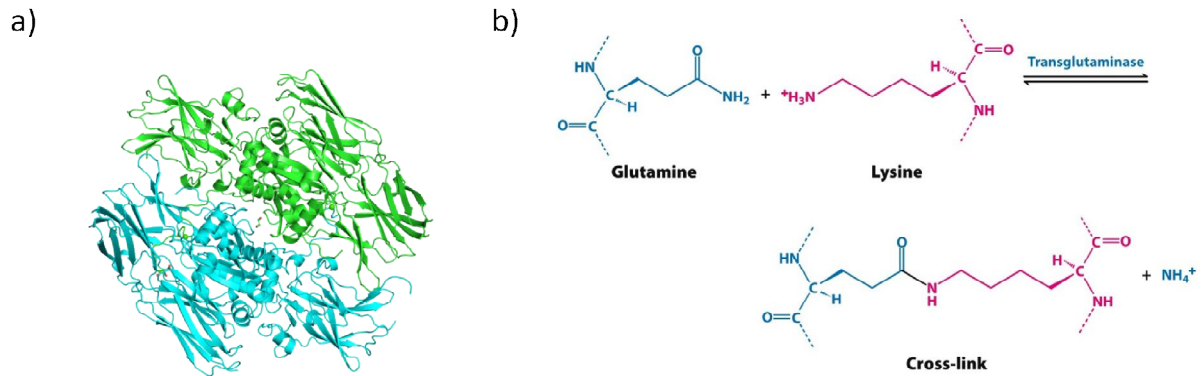


Figure 2.10: Structure and reaction mechanism of FXIIIa.

- X-ray structure of active FXIII (FXIIIa) consisting of two A subunits (blue and green) including the four major structural domains: β -sandwich domain, central catalytic core domain and two β -barrel domains (PDBe: 1EVU; Yee *et al.*, 1994).
- FXIIIa catalyzes the cross-linking reaction of fibrin clots that includes the formation of a peptide bond between lysine and glutamine side chains of different fibrin monomers (Freeman, 2012).

The diversity of bleeding disorders observed in patients with FXIII deficiency, a rare autosomal recessive syndrome, illustrated the relevance of FXIII in hemostasis (Ivaskevicius *et al.*, 2007). Life-long bleeding tendencies, declined wound healing and recurrent miscarriages are associated with FXIII deficiency (Ichinose, 2012). Mice deficient in FXIII have also been shown to have an impaired wound healing (Lauer *et al.*, 2002). FXIII mainly contributes to the prevention of fibrinolysis by fibrin stabilization and binding or incorporation of anti-fibrinolytic substrates such as α_2 -antiplasmin, TAFI and α_2 -macroglobulin within the fibrin clot (Muszbek *et al.*, 2011; Richardson *et al.*, 2013). Additionally, experimental studies demonstrated an involvement of FXIII in angiogenesis and wound healing by inducing the migration of fibroblasts and macrophages as well as fibroblast proliferation, inhibition of apoptosis and cross-linking of ECM proteins and plasma integrins (reviewed by Muszbek *et al.*, 2011). FXIII is even involved in the innate immune response against bacterial pathogens such as *S. pyogenes* by mediating the immobilization of these bacteria within fibrin networks after infection (see Figure 2.11). This entrapment process is supported by FXIII-mediated cross-linking of bacterial surface proteins with the fibrin network (Loof *et al.*, 2011b).

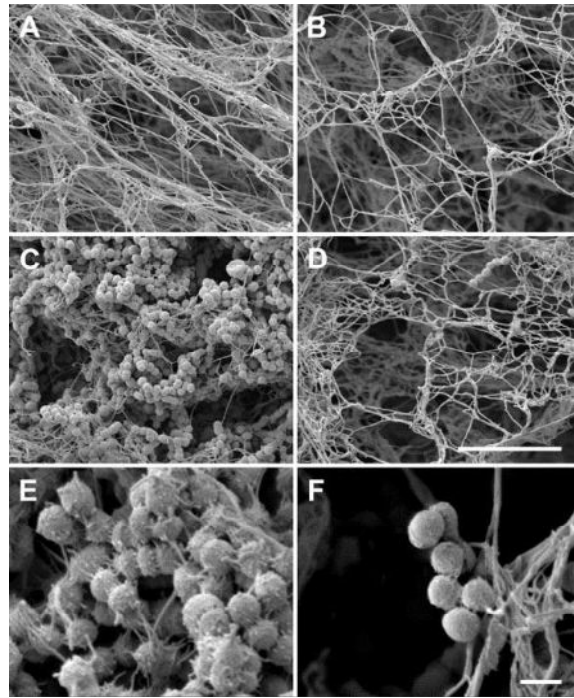


Figure 2.11: Entrapment and immobilization of *S. pyogenes* within fibrin clots.

Scanning electron micrographs showing fibrin clots generated from normal plasma (A, C and E) and FXIII-deficient plasma (B, D and F) in the absence (A and B) and presence of *S. pyogenes* AP-1 bacteria (C to F). Bacterial entrapment is mediated by FXIII resulting in tightly entwined bacteria, whereas only few bacteria are loose associated with the fibrin fibers in the absence of FXIII (Loof *et al.*, 2011b).

2.4 Aims of the study

During the last two decades there is a growing body of evidence that coagulation adopts a function within the early innate immune response against invading pathogens. Since it has been demonstrated that beside its role in hemostasis and wound healing, coagulation and especially FXIII contribute to the host defense against *S. pyogenes*, the aim of this study was the analysis of the molecular mechanisms behind these interactions. FXIII has been shown to target bacterial surface structures, such as the streptococcal M1 protein, and mediates the bacterial entrapment within fibrin clots. Accordingly, the first aim of this study was:

1. The analysis of interactions between FXIII and streptococcal surface structures.

The hypothesis about the general relevance of the FXIII-mediated entrapment as a part of the innate immune response against streptococcal infections should be proven. Thus, different bacteria belonging to the genus *Streptococcus* were analyzed for their interactions with the fibrin network and FXIII *in vitro* because the molecular mechanisms behind the interactions between streptococcal surface proteins, fibrinogen and FXIII had not been discovered yet. Therefore, the second objective of this study was:

2. The identification of streptococcal surface structures that are targeted by FXIII.

The interactions of different streptococcal M and M-like proteins with fibrinogen and FXIII should be investigated on the molecular level *in vitro*. To unravel these mechanisms, recombinant streptococcal surface proteins, *S. pyogenes* wild type and mutant strains as well as *Lactococcus lactis* mutant strains as a heterologous expression system should be used.

Since FXIII has also been shown to play a role during *S. pyogenes* infections *in vivo*, the third aim of this study was:

3. The characterization of the contribution of FXIII in the defense against streptococcal infections *in vivo*.

To this end, a murine *S. pyogenes* skin infection model using CBA wild type and FXIII-deficient mice will be employed. After subcutaneous infection with *S. pyogenes* the influence of FXIII on the development and the outcome of the infection should be investigated.

Taken together, the experiments performed in the context of this study should gain new insights into the role of the coagulation system and especially of FXIII during infections caused by streptococci. An improved understanding of these interactions might lead to the development of novel antimicrobial and therapeutic approaches against bacterial infections.

3 MATERIAL AND METHODS

3.1 Material

3.1.1 Chemicals

Table 3.1: Chemicals used in this study

Chemical/solution	Company
2-(N-Morpholino)ethansulfonic acid (MES)	Roth
5x Phusion high fidelity PCR buffer	Thermo Scientific
6x loading dye (agarose gel electrophoresis)	Fermentas
10 x FD green Buffer	Fermentas
10 x Taq Polymerase buffer	NEB
10 x T4 Ligase buffer	Fermentas
Acetic acid	Merck
Acrylamide 30% (Rotiphorese Gel 30)	Roth
Agar agar	Roth
Agarose Broad range (NEEO Ultra quality)	Roth
Amersham ECL Prime Western Blotting Detection Reagent	GE Healthcare Life Science
Ammoniumpersulfate (APS)	Serva
Ampicillin	Applichem
Bacto™ Tryptone	Becton Dickinson
Bacto™ Yeast Extract	Becton Dickinson
β Mercaptoethanol	Serva
Bovine serum albumin (BSA)	Sigma Aldrich
Bradford reagent	Sigma Aldrich
Bromophenol blue	Sigma Aldrich
Calcium chloride (CaCl ₂)	Merck, Technoclone
Chloroform	Sigma Aldrich
Columbia blood agar plates	Becton Dickinson
Coomassie Brilliant Blue R-250	Sigma Aldrich
DAPI supplemented mounting media	Molecular Probes
Dapttin® TC	Technoclone
Developer G153	AGFA
Difco™ M17 broth	Becton Dickinson

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Dimethylsulfoxid (DMSO)	Sigma Aldrich
Di-sodiumhydrogenphosphate (Na_2HPO_4)	Merck
Desoxyribonucleotides (dNTPs)	Fermentas
1-Ethyl-3-(3-dimethylaminopropyl)carbodiimide (EDC)	Roth
Erythromycin	Sigma Aldrich
Ethanol	J. T. Baker
Ethidium bromide	Applichem
Ethylenediaminetetraacetic acid (EDTA)	Roth
Fast Fixer	AGFA
Fetal calf serum (FCS)	PAA
Fluoroshield™ with DAPI	Sigma
GeneRuler Ruler™ DNA ladder mix	Fermentas
Glucose	Roth
Glycerol	Roth
Glycine	Roth
Hydrochloric acid	Roth
Imidazol	Roth
Isopropyl alcohol	Merck
Isopropyl β -D-1-thiogalactopyranoside (IPTG)	Applichem
Kanamycin	Roth
Lactose	Roth
Magnesium chloride (MgCl_2)	Finnzymes; Roth
Methanol	J. T. Baker
PAGE Ruler Prestained Protein Ladder plus	Fermentas
Paraformaldehyde (PFA) solution, 4% in PBS	USB Corporation
Ponceau S	Applichem
Skim milk powder	Sigma Aldrich
Sodium acetate	Fluka
Sodium citrate	Fluka
Sodium chloride (NaCl)	Roth
Sodium dihydrogene phosphate (NaH_2PO_4)	Roth
Sodium dodecyl sulphate (SDS)	Sigma Aldrich
Sodium hydroxide (NaOH)	Merck
Sucrose	Roth
Tetramethylethylenediamin (TEMED)	Biorad
Todd Hewitt Broth (THB)	Roth

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Tris(hydroxymethyl)-aminomethan (TRIS)	Sigma Aldrich
Triton X-100	Sigma
Trizma base	Sigma Aldrich
Tween 20	Bio-RAD
Urea	Serva

3.1.2 Expendable material and instruments

Table 3.2: Expendable material and instruments used in this study

Material/instrument	Company
aPTT measurements	
Balls micro	Merlin medical
Coagulometer MC1	Merlin medical
Cuvettes	Merlin medical
Ball dispenser	Merlin medical
Centrifugation	
Biofuge 15R	Heraeus SEPATECH
Heraeus Pico17	Thermo Electron Cooperation
iFuge MO8VT	Neuation
Sorvall® RC6 Plus	Thermo Scientific
Rotor SLA-3000	Thermo Scientific
Rotor SS-34	Thermo Scientific
5417c	Eppendorf
Dialysis	
Servpor® dialysis tubing (MWCO 12-14000; 3000)	Serva
Micro dialysis capsules QuixSep	Roth
Electrophoresis	
Agarose gel electrophoresis chamber Horizon58	Whatman
Mini Protean III™ system	Bio-RAD
Hamilton syringe	Whatman
Power supply Power PA C300	Bio-RAD
Mini-PROTEAN® TGX™ Precast Gels	Bio-RAD
Electroporation	
Electroporation cuvettes 1mm and 2 mm electrode gap	Peqlab
Gene Pulser™ and pulse controller	Bio-RAD

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Medical devices

BD Microlance™ 25G	BD Biosciences
Omnifix®-F Solo (1 mL syringe)	Braun

Microscopy

Coverslips (ø 12 mm) Thermo Scientific	Thermo Scientific
Microscope slides 76x 26 mm	Thermo Scientific
Nikon A1R confocal microscop	Nikon Instrument
Shutter HXP120C	Kübler codex
Zeiss AxioCam MRm	Zeiss
Zeiss Axio Imager A2	Zeiss
Zeiss Microscope ID03	Zeiss

Spectrometry/Photometry

Cuvettes polystyrene 10x 4x 45 mm	Sarstedt
NanoDrop® 2000c Spectrophotometer	Thermo Scientific
NOVASPEC II Spectrophotometer	Amersham Pharmacia Biotech
Sunrise™ microplate absorbance reader	Tecan

Protein purification

Amicon Ultra Centrifugal Filter devices	Sartorius
French Press FA-32	Thermo Fischer
Nickel Nitrilotriacetic acid (Ni NTA)	Qiagen
Single use plastic column	Terumo

Western Blot

ChemiDoc™ MP System	
Nitrocellulose membranes 0,2 µm	Bio-RAD
Trans-Blot SD Semi-Dry Transfer cell	Bio-RAD
Whatman filter paper	GE Healthcare Life Science

Other

Autoclave-steam sterilizer	Biomedis
Balance vicon	Acculab/Sartorius
Cell Star® 15 ml tubes Greiner bio-one	tubes Greiner bio-one
Cell Star® 50 ml tubes Greiner bio-one	Greiner bio-one
Cryo tube™ vial Nunc™	Nunc™
Dynabeads® MyOne™ Carboxylic Acid	Invitrogen
ErgoOne Pipettes (5000, 1000, 200, 20 µl, Multipipette)	Starlab

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FastPrep®-24 Instrument	MP
Immuno 96 MicroWell™ Solid Plates	Thermo Fisher Scientific
Micro tube 1.5 ml	Sarstedt
MS1 Minishaker	IKA
PCR tubes 0.2 ml	Starlab
Petri dish	Greiner bio-one
pH-meter 766	Calimatic Knick
POLYTRON PT 2100 homogenizer	KINEMATICA AG
Roller mixer SRT6	Stuart
Servpor® dialysis tubing (different MWCO)	Serva
Thermomixer 5436	Eppendorf
Tpersonal Thermocycler	Biometra
Waterbath	GFW
Zirconia/silica beads	Roth

3.1.3 Proteins and enzymes

Table 3.3: Proteins and enzymes used in this study

Protein/Enzyme	Company
<i>Bam</i> HI FD	Fermentas
Bovine serum albumin (BSA)	Bio-RAD
Coagulation factor XIII (FXIII)	Enzyme Research Laboratories
<i>Eco</i> RI FD	Fermentas
<i>Eco</i> RV FD	Fermentas
FastAP	Fermentas
Fibrogammin®P FXIII concentrate	CSL Behring
Fibrinogen (from human plasma)	Sigma
Fibrinogen-binding protein of GGS (FOG)	kindly provided by Dr. G. Gulotta and PD Dr. D.P. Nitsche-Schmitz; HZI
Hemoclot thrombin reagent	Hyphen BioMed
Lysozyme	Sigma
Mouse interleukin 6 (mIL6)	BioLegend
Mouse interleukin 10 (mIL10)	BioLegend
Phusion DNA polymerase	Finzymes/Thermo Scientific
Proteinase Inhibitor Cocktail Tablets, cComplete MINI, EDTA-free	Roche Diagnostics

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Proteinase K	Fluka
<i>Pst</i> I FD	Fermentas
Rnase (DNase free)	Applichem
<i>Sa</i> I FD	Fermentas
Streptococcal M1A3 construct	Master thesis Caroline Wesener
Streptococcal M3A1 construct	Master thesis Caroline Wesener
Streptococcal M1-2 protein	Kindly provided by Dr. G. Gulotta and PD Dr. D.P. Nitsche-Schmitz, HZI
Streptococcal M3 protein	Kindly provided by Dr. G. Gulotta and PD Dr. D.P. Nitsche-Schmitz, HZI
Streptococcal M55 protein	Kindly provided by Dr. G. Gulotta and PD Dr. D.P. Nitsche-Schmitz, HZI
T4 ligase	Fermentas

3.1.4 Antibodies and plasma

Table 3.4.: Antibodies and plasma used in this study

Antibody/serum	Dilution	Application	Source/company
Alexa 488 conjugated donkey-anti-rabbit IgG		fluorescence microscopy	Molecular Probes, Eugene, OR, USA
Alexa 647 conjugated donkey-anti-goat IgG		fluorescence microscopy	Molecular Probes, Eugene, OR, USA
Fetal calf serum (FCS)		Cultivation of pneumococci; ELISA; Immunofluores- cence microscopy	PAA
FXIII-deficient plasma		clotting assays	George King Bio- Med Inc.
goat anti-rabbit antibody Alexa 488 green conjugated	1:200	fluorescence microscopy	Abcam
goat anti-rabbit antibody HRP conjugated	1:3000	western blot	Dako
goat polyclonal anti- <i>Streptococcus pyogenes</i> group A		fluorescence microscopy	Abcam, Cambridge, UK
normal plasma		clotting assays	Healthy

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Plasminogen-deficient plasma		clotting assays	volunteers Affinity Biological Inc.
rabbit anti-fibrinogen antibody	1:10 000	western blot	Dako
rabbit polyclonal anti-factor XIIIa		fluorescence microscopy	Bioss Inc., Woburn, MA, USA
rabbit anti-streptococcal M1 protein antibody	1:3000	fluorescence microscopy	Kindly provided by Dr. S. Talay (HZI, Braunschweig)

3.1.5 Kits

Table 3.5: Kits used in this study

Kit	Company
Dneasy Blood and Tissue Kit	Qiagen
Midi Präp	Qiagen
QIAprep® Spin Miniprep Kit	Qiagen
mouse IL-6 ELISA Kit	BioLegend
PCR and Gel Clean-Up Kit	Machery-Nagel
Phire Animal Tissue Direct PCR Kit	Thermo Scientific
TOPO® TA Cloning Kit	Invitrogen

3.1.6 Antibiotics

Table 3.6: Antibiotics used in this study

Name	Application	Final concentration
Ampicillin	Cultivation of <i>E. coli</i>	100 µg/ml
Erythromycin	Cultivation of <i>S. pyogenes</i> und <i>L. lactis</i>	5 µg/ml
Kanamycin	Cultivation of <i>E. coli</i>	50 µg/ml
Spectinomycin	Cultivation of <i>S. dysgalactiae ssp. equisimilis</i>	80 µg/ml

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3.1.7 Plasmid vectors

Table 3.7: Plasmid vectors used in this study

Vector	Vector size	Selection conditions	Reference/source
pCR2.1-TOPO	3900 bp	100 µg/ml Ampicillin 50 µg/ml Kanamycin	Invitrogen
pDC _{erm}	4690 bp	500 µg/ml erythromycin	Kindly provided by Prof. V. Nizet, University of California, San Diego
pOri23		<i>E. coli</i> : 400 µg/ml Erythromycin <i>L. lactis</i> : 5 µg/ml Erythromycin	
pQE30	3460 bp	100 µg/ml Ampicillin	Qiagen

3.1.8 Oligonucleotides

Table 3.8: Oligonucleotides used in this study

Name	Sequence 5'-3'	T _m [°C]	T _a [°C]	Annotation/ reference
Primer for cloning of the B repeat region of streptococcal M1 protein (AP-1)				
B-for new2	AGTTGGGATAGAGGATCCCTTGA AAAAGAG	65,4	55	This study
B-R2-new2	CCCGTCGACTTATTCTAACTCTTT TTCTAAGACGTTAGC	69,5	55	This study
Primer for amplification of genes inserted into pCR2.1-Topo vector (TOPO cloning)				
M13 for	GTAAAACGACGGCCAG	54	60	Invitrogen
M13 rev	CAGGAAACAGCTATGAC	20	60	Invitrogen
Primer for amplification of genes inserted into pQE30				
pQE 5' Promoter SeqP (Nr. 94)	CCCGAAAAGTGCCACCTG	58	60	Primer collection, former MMIK Dept., HZI
pQE 3' SeqP (Nr. 96)	GTTCTGAGGTCATTACTGG	56	60	Primer collection, former MMIK Dep. HZI

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Primer for classification of *S. pyogenes* strains (amplification of the A region of streptococcal M1 protein)

all emmA	TATTCGCTTAGAAAATTAA	46	52	Primer collection, former MMIK Dep. HZI
all emmB	GCAAGTTCTTCAGCTTGTTT	56	52	Primer collection, former MMIK Dep. HZI

Amplification of gene sequences for truncated *emm1* gene without A region

emm_f_avrII	TGTACACCTAGGGATAGACAAAG ACTTGAAAAA	64,5	55	This study
emm_r_avrII	TCCCTAGGATTACCATCACCGTT	60,6	55	This study

3.1.9 Bacterial strains

3.1.9.1 *Streptococcus pyogenes* strains (Group A Streptococci, GAS)

Table 3.9: *S. pyogenes* strains used in this study

Strain	Serotype	Phenotype	Origin/condition	Reference/source
<i>S. pyogenes</i> 5448 (A749)	M1T1	wild type		Chatellier et al., 2000
<i>S. pyogenes</i> 5448 Δ <i>emm1</i>	M1T1	Δ M1		Lauth et al., 2009
<i>S. pyogenes</i> AP-1 (A529)	M1	wild type		Åkesson et al, 1990
<i>S. pyogenes</i> MC25	M1	Δ M1	150 µg/ml Kanamycin	Mattias Collin, Lund University, Sweden
<i>S. pyogenes</i> A600	M3	wild type		B.J. Vlamincx ; University Medical Center Utrecht, The Netherlands
<i>S. pyogenes</i> A666	M6	wild type		B. J.Vlamincx ; University Medical Center Utrecht, The Netherlands
<i>S. pyogenes</i> A304	M49	wild type	clinical skin isolate	Andreas Podbielski

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<i>S. pyogenes</i> AP-53 (A751)		wild type	Marc Walker, University of Queensland, Australia
<i>S. pyogenes</i> A856	M3.23	wild type	Strain collection former MMIK Dep. HZI

3.1.9.2 Group G Streptococci

Table 3.10: GGS strains used in this study

Strain	Serotype	Phenotype	Origin/condition	Reference/source
<i>S. dysgalactiae</i> <i>ssp. equisimilis</i> G45	StG11	Wild type		Strain collection former MMIK Dep. HZI
<i>S. dysgalactiae</i> <i>ssp. equisimilis</i> G89	StG11	Δ FOG (pJRS233 - Fog-Spc)	80 µg/ml Spectinomycin; 1 µg/ml Erythromycin	Strain collection former MMIK Dep. HZI

3.1.9.3 Other streptococci strains

Table 3.11: Other streptococci strains used in this study

Strain	Name	Serotyp	Origin/Reference
<i>Streptococcus pneumoniae</i> D39 (P35)	7466	Typ 2	NCTC, laboratory strain
<i>Streptococcus oralis</i> SV 215			Strain collection former MMIK Dep. HZI

3.1.9.4 *Escherichia coli* strains

Table 3.12: *E. coli* strains used in this study

Strain	Plasmid	Culture conditions	Reference/Source
<i>E. coli</i> DH5α			Strain collection former MMIK Dep. HZI
<i>E. coli</i> M15	pREP4	25 µg/ml Kanamycin 100 µg/ml Ampicillin	Qiagen (1994)
<i>E. coli</i> M15 + A1M3 construct	pREP4 pQE30-A1M3	25 µg/ml Kanamycin 100 µg/ml Ampicillin	Master thesis Caroline Wesener (2015)

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<i>E. coli</i> M15 + A3M1 construct	pREP4 pQE30-A3M1	25 µg/ml Kanamycin 100 µg/ml Ampicillin	Master thesis Caroline Wesener (2015)
<i>E. coli</i> M15 + A region of M1 protein (AP-1)	pREP4 pQE30-A region	25 µg/ml Kanamycin 100 µg/ml Ampicillin	Kindly provided by Dr. Giuseppe Gulotta and PD Dr. D.P. Nitsche-Schmitz, HZI
<i>E. coli</i> M15 + ABs construct of M1 protein (AP-1)	pREP4 pQE30-ABs	25 µg/ml Kanamycin 100 µg/ml Ampicillin	Kindly provided by Dr. Giuseppe Gulotta and PD Dr. D.P. Nitsche-Schmitz, HZI
<i>E. coli</i> M15 + B repeats of M1 protein (AP-1)	pREP4 pQE30-B repeats	25 µg/ml Kanamycin 100 µg/ml Ampicillin	This study
<i>E. coli</i> M15 + sCD construct of M1 protein (AP-1)	pREP4 pQE30-sCD construct	25 µg/ml Kanamycin 100 µg/ml Ampicillin	Kindly provided by Dr. Giuseppe Gulotta and PD Dr. D.P. Nitsche-Schmitz, HZI
<i>E. coli</i> TOP10			Invitrogen (1994)

3.1.9.5 *Lactococcus lactis* strains

Table 3.13: *L. lactis* strains used in this study

Strain	Plasmid	Insert/characteristics	Culture conditions	Reference
<i>L. lactis</i>	pLM1 (pVE5508 with <i>emm1</i>)	Replacement of <i>emm6</i> within pLM1	5 µg/ml erythromycin; 30°C	Cue <i>et al.</i> , 2001
<i>L. lactis</i>	pP59	Deletion of <i>emm6</i> from pVE5508	5 µg/ml erythromycin; 30°C	Cue <i>et al.</i> , 2001
<i>L. lactis</i> A1M3	pOri23-A1M3	Insertion of <i>emm3</i> gene with A region of <i>emm1</i> gene (A1M3)	5 µg/ml erythromycin; 30°C	Master thesis Caroline Wesener

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		construct)		(2015)
<i>L. lactis</i> A3M1	pOri23-A3M1	Insertion of emm1 gene with A region of emm3 gene (A3M1 construct)	5 µg/ml erythromycin; 30°C	Master thesis Caroline Wesener (2015)
<i>L. lactis</i> M3	pOri23-M3	Insertion of emm3 gene (<i>S. pyogenes</i> A856)	5 µg/ml erythromycin; 30°C	Master thesis Caroline Wesener (2015)
<i>L. lactis</i> M1ΔA	pOri23-M1ΔA	Insertion of emm1 gene without A region	5 µg/ml erythro-mycin; 30°C	This study

3.2 Microbiological methods

3.2.1 Cultivation of bacterial strains

S. pyogenes strains were cultured in THY medium and supplemented with antibiotics if it was necessary for the mutant strains. Bacterial cultures were prepared in sterile Falcon tubes and cultured overnight at 37°C and 5% CO₂ without agitation. *Streptococcus viridans* strains were cultured under similar conditions than *S. pyogenes* strains.

S. pneumoniae strains were grown in THY medium containing 1% yeast extract at 37°C and 5% CO₂, overnight without agitation.

E. coli strains were cultured in LB medium containing the appropriate antibiotics overnight at 37°C with agitation (120 rpm).

For the *L. lactis* cultivation, M17 medium supplemented with 0,5% glucose and the appropriate antibiotic was used. *Lactococci* were cultured overnight at 30°C without agitation.

For long term storage of bacteria, glycerol stocks were prepared. To this purpose 750 μ l bacterial overnight culture were mixed with 250 μ l sterile glycerol and kept at -80°C .

THY medium	30 g/l Todd Hewitt Broth
	5 g/l Bacto™ Yeast Extract *
	<i>ad 1 l</i> dH ₂ O

*for *S. pneumoniae* 10 g/l Bacto™ Yeast Extract was used

Luria Bertani (LB) medium	10 g/l Bacto™ Tryptone
	5 g/l Bacto™ Yeast Extract
	10 g/l NaCl
	<i>ad</i> 1 l dH ₂ O

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M17 medium	37.25 g/l Difco™ M17 broth powder
	950 ml dH ₂ O
	50 ml 10% Lactose (added after sterilization and cooling to 50°C)

For preparation of the corresponding agar plates, 15 g/l of agar agar was added to the medium before autoclaving.

3.2.2 Monitoring of bacterial growth and adjusting of bacterial suspensions

Bacterial growth was monitored by measuring the optical density at a wavelength of 600 nm (OD₆₀₀) using a spectrophotometer.

To adjust comparable bacterial amounts, especially for experiments with streptococci, the transmission at a wavelength of 600 nm (T₆₀₀) was measured. Bacterial concentrations were adjusted taking into account that a T₆₀₀ of 10% corresponds to a bacterial concentration of approximately 5×10^8 cfu/ml.

3.2.3 Preparation and transformation of competent bacteria

Transformation was used to insert foreign DNA or artificial DNA constructs into the desired bacterial strains. This method uses the property of certain bacteria, like *E. coli*, to achieve foreign DNA by horizontal gene transfer.

3.2.3.1 Preparation of chemical competent *E. coli* cells

Initially, 100 ml LB medium supplemented with appropriate antibiotics were inoculated with an overnight culture of the relevant *E. coli* strain in a ratio 1:100. Bacteria were grown up to OD₆₀₀ between 0.4 and 0.45 at 37°C and 120 rpm. After 10 min incubation on ice, cultures were centrifuged for 10 min at 5 000 rpm and 4°C. The supernatant was discarded and the pellet was resuspended in 50 ml ice cold MgCl₂. After centrifugation for 10 min at 5 000 rpm and 4°C, the pellet was resuspended in 50 ml ice cold CaCl₂. After another centrifugation step (see above), the bacterial pellet was resuspended in 4 ml ice cold CaCl₂/Glycerol solution and incubated on ice for 20 min. Cell suspension was aliquoted to 200 µl, snap frozen in liquid nitrogen and stored at -80°C until use.

MgCl₂	100 mM
CaCl₂	50 mM
CaCl₂/Glycerol	50 mM CaCl ₂ 12,5 % (V/V) Glycerol

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3.2.3.2 Transformation of chemically competent *E. coli* cells

Briefly, cells were thawed on ice and 2 µl of plasmid DNA / 5 µl ligation reactions were added. The mixture was incubated on ice for 15 to 30 min and subjected to a heat shock for 30 s at 42°C. Bacteria were resuspended in 950 µl of LB medium without antibiotics and further incubated for at least 45 min at 37°C and 450 rpm. At the end, 50 to 100 µl of bacterial suspension were plated on selective LB agar plates containing the appropriate antibiotic and incubated overnight at 37°C.

After transformation of ligation products, the selected bacterial colonies, probably carrying the desired insert, were checked by colony PCR (see section 3.3.4.2) and DNA sequence analysis (see 3.3.10).

3.2.3.3 Preparation of electro competent *E. coli* cells

E. coli was grown overnight in LB medium and a subculture was prepared at a ratio of 1:100 in 500 ml LB medium containing the appropriate antibiotic. Bacteria were then grown at 37°C and 120rpm to a maximum OD₆₀₀ of 0.6 and incubated on ice for 15 to 30 min. After centrifugation for 10 min at 5 000 rpm and 4°C, cells were washed with 250 ml ice-cold water followed by another washing step with 250 ml ice-cold water containing 10% glycerol. Finally, the bacterial pellet was resuspended in 1 ml of 10% (V/V) glycerol and incubated on ice for 5 min. Bacterial suspensions were aliquoted, snap frozen in liquid nitrogen and stored at -80°C until use.

3.2.3.4 Transformation of electrocompetent *E. coli* cells

Bacterial suspensions were mixed with 1 µl plasmid DNA / 2 – 5 µl ligation products and transferred to precooled electroporation cuvettes. Electrocompetent cells were transformed by electroporation with 2.5 kV, 25 µF and 200 Ω followed by rapid addition of 250 µl LB medium. Cells were incubated at 37°C and 120 rpm for at least 45 min, followed by plating on selective LB agar plates containing the appropriate antibiotic. Bacterial colonies were checked for inserted DNA as described above.

3.2.3.5 Preparation of competent *L. lactis* cells

To prepare competent *L. lactis* cells, 45 ml of GM17 medium containing 1% glycine were inoculated with 5 ml of an overnight culture. Cells were grown to an OD₆₀₀ of 0.3, spun down for 10 min at 4 000 rpm and 4°C and pelleted bacterial cells were washed three times with 20 ml of a sucrose/glycerol solution. Between each washing step, cells were centrifuged for 10 min at 4 000 rpm and 4°C. Finally, cells were resuspended in 250 µl sucrose/glycerol solution, aliquoted and either directly used for transformation or snap frozen in liquid nitrogen and stored at -80°C until use.

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GM17 medium	18.63 g M17 broth 475 ml dH ₂ O 25 ml sterile 10% (w/V) glucose (added after sterilization and cooling to 50°C)
GM17 + 1 % glycine	18.63 g M17 broth 5 g glycine 475 ml dH ₂ O 25 ml sterile 10% (w/V) glucose (added after sterilization and cooling to 50°C)

For preparing the corresponding agar plates, 15 g/l of agar agar was added to the medium before autoclaving.

Sucrose/glycerol solution	0.5 M Sucrose 10 % (V/V) glycerol
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3.2.3.6 Transformation of competent *L. lactis* cells

A volume of 50 µl competent *L. lactis* bacteria was carefully mixed with 0.5 – 1 µg of DNA on ice. Bacteria were transformed by electroporation using 2000 V. Immediately after electroporation, 750 µl recovery buffer were added and the mixture was incubated at 30°C for at least 2 h. Finally, cells were plated on GM17 plates containing two different concentrations of the appropriate antibiotic (in this study 3 and 5 µg/ml erythromycin) and incubated at 30°C for a period of 2 to 3 days.

Recovery Buffer	GM17 0.5 M Sucrose 20 mM MgCl ₂ 2 mM CaCl ₂
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3.2.4 Immobilization of *S. pyogenes* within the fibrin network

S. pyogenes was cultured as described above (see 3.2.1). For immobilization experiments 5×10^5 cfu bacteria in a volume of 100 µl sodium citrate buffer were mixed with 100 µl of normal or FXIII-deficient human plasma. Clotting was induced by the addition of 50 µl Hemoclot Thrombin reagent. Clots were then covered with 1 ml of 1% normal or FXIII-deficient human plasma and 50 µl of the supernatant were immediately plated onto blood agar in 10 fold serial dilutions. The number of bacteria was determined by counting colonies after 18 h of incubation at 37°C and 5% CO₂.

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Sodium citrate

12,9 mM

3.3 Molecular biology methods

3.3.1 Isolation of chromosomal DNA from *S. pyogenes*

To isolate chromosomal DNA from *S. pyogenes*, 10 – 15 ml of an overnight culture were centrifuged for 7 min at 3 700 x g. The resulting bacterial cell pellet was resuspended in 180 µl TE buffer and 20 µl of DNase-free RNase ($c_0 = 10 \mu\text{g/ml}$) were added. The solution was transferred to ca. 250 µl sterile zirconia beads and cells were lysed by using a cell homogenizer for 30 s with a velocity of 4 m/s. After addition of 200 µl of buffer AL, the beads were spun down for 30 s. The lysate was subsequently transferred into a new reagent tube and the zirconia beads were discarded. To remove the remaining proteins, 25 µl of proteinase K were added, tubes were gently inverted and incubated at 56°C for 30 min. To precipitate the DNA, 200 µl of ethanol (96 – 100%, p.a.) were added and the complete mixture was applied to a DNeasy Mini spin column. After centrifugation for 1 min at 8 000 rpm, the flow-through was discarded and the retained DNA was washed by adding 500 µl of buffer AW1 and centrifuging for 1 min at 14 000 rpm. The flow-through was discarded and the membrane was dried by centrifuging for 1 min at 14 000 rpm. Finally, DNA was eluted by the addition of 200 µl buffer AE followed by a 5 min incubation step and centrifugation for 1 min at 8 000 rpm. The quality of DNA was verified by agarose gel electrophoresis (see 3.3.3).

Buffer AL, AW1, AE and DNeasy Mini spin columns were provided with the DNeasy Blood & Tissue Kit by Qiagen.

TE buffer

10 mM TRIS/HCl

1 mM EDTA

pH8.0

3.3.2 Isolation of plasmid DNA

Plasmid DNA from *E. coli* cells was prepared by using the QIAprep® Spin Miniprep kit according to the manufacturer's instructions. Isolated DNA was analyzed by agarose gel electrophoresis (see section 3.3.3) and stored at -20°C until use.

3.3.3 Agarose gel electrophoresis

Agarose gel electrophoresis was used for quantitative and qualitative analysis of DNA molecules after plasmid and chromosomal DNA isolation. For preparation of agarose gels, the appropriate amount of agarose was solved in 1 x TAE by boiling. DNA samples were

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mixed with 6 x DNA loading dye and loaded on the gel. GeneRuler™ DNA ladder mix was used as a molecular weight standard and 1 x TAE was used as running buffer. Electrophoresis was performed for approximately 40 – 60 min at 100 V. Agarose gels were incubated in an ethidium bromide solution (1 µg/ml in H₂O) for 10 min and the DNA was visualized by using an UV-transilluminator or a Bio-RAD GelDoc station.

TAE (Tris Acetate EDTA)	2 M Tris/HCl,	pH 8.5
	1 M Acetic acid	
	100 mM EDTA	

3.3.4 Polymerase chain reaction (PCR)

3.3.4.1 Standard PCR

The standard PCR protocol was used to amplify defined DNA fragments out of a longer template DNA (chromosomal DNA, plasmid DNA) and to introduce specific restriction sites into defined genes to enable whose cloning into plasmid vectors.

Standard PCR Protocol

Table 3.16: Standard PCR protocol

Reagent	Stock concentration	Final concentration/ amount
Template DNA		100 – 500 ng
Phusion PCR buffer with MgCl ₂	5 x	1 x
dNTPs	2 mM	200 µM
Primer forward	10 pmol/µl	300 nM
Primer reverse	10 pmol/µl	300 nM
Phusion high fidelity DNA polymerase	2 U/µl	0,04 U/µl
MQ H ₂ O		ad 50 µl

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Standard PCR program

Table 3.17: Standard PCR program

Reaction step	Temperature	Time
Initial denaturation	98°C	1 min
Denaturation	98°C	30 s
Annealing	T _a	30 s
Elongation	72°C	t _e
Final elongation	72°C	5 min
	4°C	∞

T_a: Annealing temperature, primer specific

t_e: Elongation time, dependent on the length of the desired PCR product and the processivity of the polymerase which is used (Phusion polymerase ~1 kb/30 sec, Taq polymerase ~1 kb/1 min)

3.3.4.2 Colony PCR

Colony PCR was the method of choice to check bacterial clones for the desired DNA insert after ligation, transformation and antibiotic selection. For this purpose a single bacterial colony was resuspended in 50 µl sterile MQ water and the cells were lysed by boiling for 10 min at 95°C. The released DNA was used as a template for PCR which was performed according the protocol described below (Table 3.18).

Table 3.18: Colony PCR protocol

Reagent	Stock concentration	Final concentration/amount
Template DNA		2 µl
Taq PCR buffer	10 x	1 x
MgCl ₂	25 mM	2.5 mM
dNTPs	2 mM	200 µM
Primer forward	10 pmol/µl	300 nM
Primer reverse	10 pmol/µl	300 nM
Taq DNA polymerase	2 U/µl	0.05 U/µl
MQ H ₂ O		ad 20 µl

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Table 3.19: Colony PCR program

Reaction step	Temperature	Time	
Initial denaturation	95°C	1 min	
Denaturation	94°C	15 s	} 30 x
Annealing	T _a	30 s	
Elongation	72°C	t _e	
Final elongation	72°C	5 min	
	4°C	∞	

3.3.5 Determination of DNA concentration

The A₂₆₀ was measured using the Nanodrop spectrophotometer and DNA content was calculated by stating that an A₂₆₀ of 1,0 at 260 nm corresponds to a concentration of 50 µg/ml of double-stranded DNA.

3.3.6 Specific restriction of DNA

Restriction reactions were performed according to the protocol below (Table 3.20) for 3 h or overnight at 37°C. In some cases, it was necessary to inactivate the restriction enzymes after the reaction following the manufacturer's instructions.

Table 3.20: Specific restriction protocol

Reagent	Stock concentration	Final concentration/ amount
DNA (Plasmid DNA or PCR product)	various	1 µg
Restriction buffer	10 x	1 x
Restriction endonuclease I		1 µl/1 µg DNA
Restriction endonuclease II		1 µl/1 µg DNA
(if necessary)		
MQ H ₂ O		ad 50 µl

3.3.7 Dephosphorylation of DNA after specific restriction

Alkaline phosphatase (AP) dephosphorylates the 5' terminus of vector DNA and thus avoids re-ligation of linearized plasmid vector DNA after specific restriction. 1 U of AP was added after specific restriction and samples were incubated at 37°C for 1 h.

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3.3.8 Ligation

Ligation is the method of choice to covalently link DNA molecules and is mainly used to insert specific DNA fragments into distinct plasmid vectors. This reaction is catalyzed by T4 DNA ligase which connects free 3' hydroxy groups with free 5' phosphate groups by formation of a phosphodiester bond. For ligation insert DNA and vector DNA, after specific restriction, were used in a ratio of 5:1 or 3:1 (see Equation 3.1). Reactions were prepared according to the protocol below (Table 3.21) and incubated overnight at 4 or 16°C. Ligation products were either directly used to transform competent bacteria or stored at – 20°C.

Table 3.21: Ligation protocol

Reagent	Stock concentration	Final concentration/amount
T4 Ligase buffer	10 x	1 x
Vector DNA	Various	25 ng
Insert DNA	Various	x
T4 DNA Ligase	5 U/μl	2.5 U
MQ H ₂ O		ad 20 μl

The amount of insert DNA which is used for ligation into a distinct plasmid vector was calculated using the following equation (Mülhardt, 2006):

Equation 3.1: Ligation ratios

$$\text{Amount of insert DNA [ng]} = \frac{R \times \text{amount of vector DNA [ng]} \times \text{size of insert DNA [bp]}}{\text{size of vector DNA [bp]}}$$

R: dependent on the ratio between insert and vector DNA (5:1 or 3:1)

3.3.9 Purification of PCR products and DNA from agarose gels

PCR products and DNA bands were cut off from agarose gels and purified using the PCR and Gel Clean-Up Kit by Machery-Nagel according to the manufacturer's protocol.

3.3.10 DNA sequencing

The DNA sequence of the generated DNA constructs was checked by DNA sequencing performed by the HZI sequencing service (Group GMAK) using the chain-terminating method (Sanger *et al.*, 1977). DNA sequence data was evaluated using Bioedit software.

3.3.11 Desalting of DNA solutions by micro dialysis

A small petri dish was filled up with sterile MQ water and a membrane filter (pore size: 0.025 μm) was placed on the water surface. The DNA solution was then carefully pipetted

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onto the membrane filter and incubated for 30 min at RT. The DNA solution was removed from the filter and used to transform electro competent *E. coli* cells.

3.3.12 TOPO cloning method

The TOPO cloning strategy comprises three successive steps. In a first reaction adenosine (A) residues were added to the desired PCR product. For that purpose, the following reaction was prepared (Table 3.22) and incubated at 72°C for 10 min.

Table 3.22: TOPO cloning protocol – addition of A residues

Reagent	Volume
PCR product	10 ng / ca. 2 µl
10 x Taq polymerase reaction buffer	0.4 µl
dNTPs (or dATPs)	0.1 µl
Taq DNA polymerase	0.25 µl
ddH ₂ O	1.25 µl

Afterwards the TOPO cloning reaction was performed following to the protocol below (Table 3.23). The reaction was incubated at RT for 1 h. Mixtures were then stored on ice until transformation of chemical competent *E. coli* TOP10 cells (see section 3.2.3.2).

Table 3.23: TOPO cloning protocol – TOPO cloning reaction

Reagent	Volume
PCR product with A overhangs	2 µl
Salt solution	1 µl
TOPO vector	0.5 µl
ddH ₂ O	ad 6 µl

3.4 Protein chemistry methods

3.4.1 Recombinant protein production in *E. coli*

The QIAexpress system was used for recombinant protein production. This heterologous expression system offers high-level production of target proteins containing a hexa-histidin tag (6x His tag) in *E. coli*. To this purpose, the desired DNA constructs were inserted into the multiple cloning site (MCS) of the appropriate pQE30 plasmid vector. To effectively regulate the extremely high transcription rate, *E. coli* M15 cells were used as host strains for

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recombinant gene expression. These cells contain, beside the described expression plasmid (pQE30), the plasmid pREP4 which constitutively expresses the lac repressor protein for transcription repression. Therefore, gene expression can be induced rapidly and tightly controlled by the addition of isopropyl- β -D-thiogalactoside (IPTG) that binds to the repressor, leading to the release from the promotor to enable the binding of RNA polymerase and transcription.

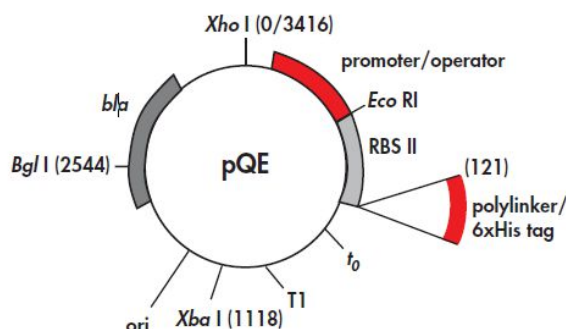


Figure 3.1: Vector map of pQE30.

The pQE plasmid vector contains different restrictions sites (*Bgl*I, *Xho*I, *Eco*RI, *Xba*I), a ribosomal binding site (RBSII), an antibiotic selection marker (gene for β -lactamase for ampicillin resistance), an origin of replication (ori), a promotor/operator region and the DNA sequence for a histidine tag (6x His tag) (adapted from “The QIAexpressionist” manual, Qiagen, 06/2003).

3.4.1.1 Test of recombinant gene expression

A volume of 20 ml of LB medium supplemented with ampicillin and kanamycin were inoculated from an overnight culture of *E. coli* M15 in a 1:100 ratio and incubated at 37°C and 120 rpm. IPTG was added (final concentration 1 mM) when the bacterial culture reached an OD₆₀₀ of 0.5 – 0.8 to induce gene expression. Optimal gene expression was determined by growing the bacteria for 4 to 6 h at different temperatures (e. g. 25°C, 30°C and 37°C). A 250 μ l sample was taken from each culture hourly and immediately centrifuged for 5 min at 10 000 rpm. Cells were lysed by resuspension in urea buffer and by addition of 5 x SDS sample buffer. Gene expression was analyzed by SDS PAGE.

Urea buffer	8 M Urea
	100 mM Na ₂ HPO ₄
	10 mM TRIS/HCl

3.4.1.2 Recombinant gene expression

For the expression of desired DNA constructs, a specific volume of LB medium supplemented with ampicillin and kanamycin as well as 0.05 % (V/V) glucose was inoculated

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with a *E. coli* M15 overnight culture in a 1:100 ratio. Cells were grown to an OD₆₀₀ of 1.0 – 1.2 at 37°C with agitation and gene expression was induced by the addition of IPTG (final concentration 1 mM) under the determined optimal conditions. Cells were harvested by centrifugation for 20 min at 4 500 rpm and 4°C (rotor SA-3000), the wet weight of the bacterial cells was determined and the bacterial cell pellet was stored at -20°C until use.

3.4.2 Purification of recombinant proteins

In this study proteins of interest (POI) were purified by immobilized metal chelate affinity chromatography (IMAC), a common method to purify his-tagged POIs with high homogeneity. For purification of soluble proteins, bacterial pellets were resuspended after recombinant expression in 5 ml of purification buffer I per gramm of bacterial wet weight and cells were disrupted by using a high-pressure cell homogenizer (french press). A protease inhibitor mix was added to prevent degradation of proteins after cell lysis. Bacterial cells were disrupted by pressing three times. To separate cell debris from soluble proteins, cell lysates were centrifuged for 30 min a 12 000 rpm and 4°C (rotor: SS-34). Ni-NTA agarose was resuspendend and 6 ml were pipetted into a fresh single use plastic column. The Ni-NTA matrix was equilibrated with 5 – 10 column volumes (CV) purification buffer I. The supernatant containing the soluble protein fraction including the POI was loaded onto the Ni-NTA matrix and the protein mixture was allowed to slowly pass through the purification matrix and the 6 x His-tagged POI to bind to the matrix. This loading step was repeated twice. The flow-through was collected and stored at 4°C. *E. coli* proteins which bound unspecificly to the Ni-NTA matrix by natural histidine residues were removed by washing four times with 5 ml purification buffer II which contained low imidazole concentrations. Washing fractions were collected and stored at 4°C. Elution of the POI from the Ni-NTA matrix was performed using high imidazole concentrations. To this end, 3 ml of purification buffer III were pipetted onto the Ni-NTA matrix, incubated for 5 min and allowed to pass through the Ni-NTA matrix. The flow-through of this step (eluate) contains the POI. The elution step was repeated twice. Elution fractions were collected and stored at 4°C. The success of protein purification was determined by SDS-PAGE analysis.

Purification Buffer I	50 mM NaH ₂ PO ₄	
	300 mM NaCl	pH 8.0
Purification Buffer II	50 mM NaH ₂ PO ₄	
	300 mM NaCl	
	20 mM Imidazol	pH 8.0

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Purification Buffer III	50 mM NaH ₂ PO ₄	
	300 mM NaCl	
	250 mM Imidazol	pH 8.0

3.4.3 SDS polyacrylamide gel electrophoresis

Complex protein mixtures and protein sizes were analyzed by sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) according to the method of Laemmli (Laemmli, 1970). Protein samples were prepared by boiling at 95°C for 5 min in SDS sample buffer and then loaded on the SDS gel. For electrophoresis, a constant current of 20 mA/gel was set up for protein migration through the stacking gel and 30 mA/gel were used after samples had reached the resolving gel. After electrophoresis gels were stained with Coomassie solution and proteins bands were visualized after destaining.

5 x sample buffer (reducing)	100 mM TRIS/HCl	pH 8
	16% (v/v) Glycerol	
	4.8% (w/v) SDS	
	0.1% (w/v) Bromine phenol blue	
	2% (v/v) β mercapto ethanol	
Resolving gel buffer	1.5 M TRIS/HCl	pH 8.8
Stacking gel buffer	0.5 M TRIS/HCl	pH 6.8
SDS Running buffer	250 mM TRIS/HCl	
	1.9 M Glycine	
	1% (w/v) SDS	
	ad 1 l MQ H ₂ O	
Coomassie Staining solution	40% (V/V) Methanol	
	10% (V/V) Acetic acid	
	1 g/l Coomassie Brilliant blue R250	
	ad 1l MQ H ₂ O	
Destaining solution	40% (V/V) Methanol	
	10% (V/V) Acetic acid	
	ad 1l MQ H ₂ O	

For the preparation of polyacrylamide gels the following protocols were used (see Table 3.24 and Table 3.25).

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Table 3.24: Preparation of polyacrylamide separation gels

8% acrylamide	Volume for 2 Gels	Volume for 4 Gels
	[ml]	[ml]
Water	4.6	9.3
30% Acrylamide mix	2.7	5.3
Resolving gel buffer	2.5	5
10% (w/V) SDS	0.1	0.2
10% (w/V) APS	0.1	0.2
TEMED	0.01	0.02
10% acrylamide		
Water	4	7.9
30% Acrylamide mix	3.3	6.7
Resolving gel buffer	2.5	5
10% (w/V) SDS	0.1	0.2
10% (w/V) APS	0.1	0.2
TEMED	0.008	0.012
12% acrylamide		
Water	3.3	6.6
30% Acrylamid-Mix	4	8
Resolving gel buffer	2.5	5
10% (w/V) SDS	0.1	0.2
10% (w/V) APS	0.1	0.2
TEMED	0.08	0.016

Table 3.25: Preparation of polyacrylamide stacking gels

Stacking gel	Volume for 2 Gels	Volume for 4 Gels
	[ml]	[ml]
Water	2.7	5.5
30% Acrylamide mix	0.67	1.3
Stacking gel buffer	0.5	1
10% (w/V) SDS	0.04	0.08
10% (w/V) APS	0.04	0.08
TEMED	0.08	0.016

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3.4.4 Western Blot

The method of choice for specific detection of proteins out of complex mixtures was the western blot technique. To this end, protein samples separated by SDS PAGE were transferred to a nitrocellulose membrane by blotting. In this study, a semi-dry blotting system was used. For this purpose, six pieces of Whatman paper, the nitrocellulose membrane and the SDS polyacrylamide gel, after electrophoresis, were equilibrated in 1 x blotting buffer and arranged in a “sandwich”-like manner. Blotting was performed for 20 min with a constant voltage of 20 V. Free protein binding site on the membranes were then blocked in 1xPBST containing 5% skim milk for at least 1 h at RT or overnight at 4°C. After three washing steps with 1x PBST for 5 min at RT, the primary detection antibody, directed against the POI was added and the membrane was incubated for at least 1 h at RT or alternatively overnight at 4°C. This incubation step was followed by three washing steps performed as before and incubation of the membrane with the secondary antibody, which is directed against the primary antibody and conjugated with horseradish peroxidase (HRP), for 1 to 2 h at RT. The blot was developed by using electro-chemiluminescence ECL detection reagent, which contains luminol and hydrogen peroxide. Chemiluminescence is produced by oxidation of luminol by HRP conjugated to the secondary antibody and can be detected by using the ChemiDoc station or by developing and fixing on chemiluminescence films.

10 x blotting buffer	58 g/l TRIS-Base 29 g/l Glycin 3.7 g/l SDS	
1 x blotting buffer	diluted from 10x stock solution ad 800 ml with MQ water + 20% (V/V) methanol ad 1 l	
1 x PBST	1 x PBS 0.05% Tween (V/V)	
20 x PBS	160 g/l NaCl 4 g/l KCl 15.2 g/l Na ₂ HPO ₄ · 2 H ₂ O 4 g/l KH ₂ PO ₄	pH 7.4

3.4.5 Fibrinogen pulldown assay

To determine the fibrinogen-binding capacity of proteins, a fibrinogen pulldown assay was established in this study. The principle of this method is based on the coupling of the investigated proteins to carboxy-activated magnetic *Dynabeads* followed by the incubation

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with fibrinogen. By applying a magnetic field, it is possible to isolate fibrinogen bound to proteins which were coupled to magnetic beads.

For this assay, POIs were dialysed against a low pH MES buffer and protein concentration was determined. The assay comprises three steps: 1. Activation of magnetic *Dynabeads* with 1-Ethyl-3-(3-dimethylaminopropyl)-carbodiimide (EDC); 2. Ligand immobilization; and 3. Isolation of the target protein by the fibrinogen pulldown. For activation, 100 μ l *Dynabeads* (c_0 = 10 mg/ml) were incubated with 100 μ l of 10 mM NaOH for 5 to 10 min. NaOH was removed after applying the magnetic beads to a magnet rack followed by three washing steps with 100 μ l ddH₂O. During the last washing step, EDC was dissolved in cold ddH₂O to a concentration of 20 mg/ml, 67 μ l were added to the *Dynabeads* and the reaction was incubated for 30 min at 4°C with slow tilt rotation. The EDC solution was then removed as described above and activated beads were washed with 100 μ l ddH₂O followed by 100 μ l MES buffer as quickly as possible to avoid hydrolysis of activated carboxy groups. To couple the desired ligand to the *Dynabeads*, 20 μ g of ligand protein were added to the *Dynabeads* and incubated at for 30 min at RT or for 2 h at 4°C with slow tilt rotation. After ligand immobilization, beads were washed three times with 300 μ l PBS as described before. The last step of the assay was the isolation of the target protein by a fibrinogen pulldown. To this purpose proteins-coupled *Dynabeads* were incubated with 50 μ g fibrinogen for 30 min at RT or 2 h at 4°C with slow tilt rotation. After three washing steps with 300 μ l PBS, fibrinogen was eluted from the beads by resuspension in 5 x SDS sample buffer and boiling for 5 min at 95 °C. After centrifugation for 5 min at 13 000 rpm, samples were analyzed by SDS-PAGE.

MES buffer	25 mM MES	pH 6.0
	250 mM NaCl	

3.4.6 Quantification of proteins

3.4.6.1 Protein quantification by Bradford assay

To prepare a calibration curve for the Bradford assay, serial dilutions of BSA protein ranging from 0 to 500 μ g were prepared in PBS (0, 75, 100, 200, 250, 300, 400 and 500 μ l/ml BSA). A volume of 10 μ l of each BSA concentration was mixed with Bradford reagent, incubated for 2 to 10 min at RT in the dark and the $A_{595\text{nm}}$ was determined. The same procedure was applied for the investigated protein sample. Three replicates of each concentration were measured for generating the calibration curve. Two replicates of each protein sample were measured. Protein concentrations were calculated using the measured A_{595} data and the linear equation of the calibration curve.

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3.4.7 Dialysis

Dialysis is a technique to desalt and to exchange buffers in protein solutions. For this purpose Servpor® dialysis tubes with a molecular weight cut off (MWCO) corresponding to the molecular weight of the POI were used. Dialysis tubes were soaked in MQ water for at least 30 min at RT and protein solutions were filled into dialysis tubes and dialysis was performed at 4°C against the 10-fold volume of the final buffer with slow stirring.

3.4.8 Concentration of protein solutions

For some experiments, high protein concentrations were needed. To increase the concentration of protein solutions after purification, Amicon Ultra Centrifugal Filter devices with the appropriate MWCO were used. First, centrifugation devices were equilibrated with the storing buffer of the protein by adding 10 ml buffer and centrifugation at 4 000 – 5 000 rpm until the buffer was completely flowed through. To increase the protein concentration, protein solutions were loaded onto the centrifugation device and the volume was continuously reduced by centrifugation.

3.5 Biochemical methods

3.5.1 Measurement of activated partial thromboplastin time (aPTT)

Activation of the intrinsic pathway of coagulation was determined by measuring the activated partial thromboplastin time (aPTT) in murine plasma using a coagulometer. To this end, 50 µl of citrated plasma obtained from uninfected and *S. pyogenes*-infected mice were incubated for 60 s at 37°C, 50 µl Kaolin were added followed by another incubation step for 60 s at 37°C. Clotting was then initiated by the addition of 50 µl CaCl₂ solution (25 mM) and the coagulation time was measured.

3.5.2 Fibrinogen conversion experiments

3.5.2.1 Fibrinogen conversion experiments using purified proteins

To investigate the final step of the clotting reaction *in vitro*, the conversion of fibrinogen to fibrin was examined. For this purpose, 20 µg purified fibrinogen, 2 µl thrombin reagent and 5 µg recombinant FXIII were mixed in a total volume of 60 µl and incubated at 37°C for 2 h. Reactions were then supplemented with 5 x SDS sample buffer and boiled for 5 min at 95°C. Fibrinogen conversion was followed by SDS PAGE and western blot analysis. To investigate the influence of several streptococcal M proteins and M protein variants on fibrinogen conversion, 0.5 µg or 5 µg of the desired protein were added to the described reactions. Sodium citrate (12.9 mM) was used as buffer system for all reactions.

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Reactions for fibrinogen conversion experiments (total volume/reaction: 60 µl):

- | | | |
|---|---|----------|
| 1.) Fibrinogen | } | controls |
| 2.) Fibrinogen, Thrombin | | |
| 3.) Fibrinogen, Thrombin, FXIII | | |
| 4.) Fibrinogen, FXIII | | |
| 5.) Fibrinogen, Thrombin, 5 µg M protein variant | | |
| 6.) Fibrinogen, Thrombin, 5 µg M protein variant, FXIII | | |
| 7.) Fibrinogen, Thrombin, 0.5 µg M protein variant | | |
| 8.) Fibrinogen, Thrombin, 0.5 µg M protein variant, FXIII | | |
| 9.) 5 µg M protein | | |

3.5.2.2 Fibrinogen conversion experiments in the presence of bacteria

S. pyogenes or *L. lactis* strains were cultured overnight and adjusted to a T_{600} of 10%. For each reaction, 3 ml of bacterial suspension were centrifuged for 4 min at 8 000 rpm and the bacterial pellet was resuspended in 100 µl 12.9 mM sodium citrate buffer. Reactions were prepared as following:

- | | | | | |
|----|--------|--|---|----------|
| 1. | 100 µl | Bacterial suspension | } | controls |
| | 100 µl | Fibrinogen (final concentration 0.3 mg/ml) | | |
| | 5 µl | Thrombin | | |
| 2. | 100 µl | Bacterial suspension | | |
| | 100 µl | Fibrinogen (final concentration 0.3 mg/ml) | | |
| | 5 µl | Thrombin | | |
| | 10 µl | FXIII (final concentration 0.05 mg/ml) | | |
| 3. | 100 µl | Bacterial suspension | | |
| | 100 µl | Fibrinogen (final concentration 0.3 mg/ml) | | |
| 4. | 35 µl | Fibrinogen (final concentration 0.3 mg/ml) | | |
| | 2 µl | Thrombin | | |
| | 23 µl | Sodium citrate | | |
| 5. | 35 µl | Fibrinogen (final concentration 0.3 mg/ml) | | |
| | 2 µl | Thrombin | | |
| | 5 µl | FXIII (final concentration 0.05 mg/ml) | | |
| | 23 µl | Sodium citrate | | |

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After incubation for 30 min at 37°C, 5 x SDS sample buffer was added and samples were boiled at 95°C for 10 min. Fibrinogen conversion was followed by SDS PAGE and western blot analysis.

3.6 Mouse strains and infection model

3.6.1 Mouse strains

Wild type and FXIII-deficient mice in the CBA/Ca background were used in this study. CBA/Ca wild type mice were purchased from Harlan (Venray, The Netherlands). FXIII-deficient animals (FXIII^{-/-}) were kindly provided by CSL Behring (Marburg, Germany). Mice were housed in a pathogen-free animal facility at the Helmholtz Centre for Infection Research and maintained under standard conditions according to institutional guidelines. All experiments were approved by the appropriate ethical committee for animal experimentation (Niedersächsisches Landesamt für Verbraucherschutz und Lebensmittelsicherheit, Oldenburg, Germany; permit 33.9-42502-04-12/1009).

3.6.2 Animal infection model

For *in vivo* experiments, 8 to 12 week old female wild type or FXIII^{-/-} mice were subcutaneously inoculated with 5 x 10⁷ CFU *S. pyogenes* strain 5448 on the back. In some experiments, mice were treated with 200 U/kg body weight of a human FXIII-concentrate Fibrogammin®P subcutaneously at the site of infection 1 h after bacterial inoculation. Mice were monitored and weighted daily after infection and mortality was recorded for generating survival curves. At indicated time points mice were sacrificed, citrated blood was drawn by cardiac puncture and centrifuged for 10 min at 5000 rpm to prepare plasma for immunological approaches or for the measurement of clotting times. Skin biopsies from the local focus of infection were obtained for histological examination or double immunofluorescence analysis. Spleens and livers were dissected for the determination of bacterial loads at 72 h of infection. For this purpose, spleens and livers were homogenized in 5 ml of PBS using a Polytron disperser and 10-fold serial dilutions of the organ homogenates as well as blood samples were plated onto blood agar. The number of bacteria was determined by counting colonies after 18 h of incubation at 37°C.

3.7 Immunological methods

3.7.1 Enzyme-linked immunoabsorbant assay (ELISA)

3.7.2 Interleukin-6 ELISA

The level of IL-6 in the plasma of uninfected or *S. pyogenes*-infected mice was measured by using the Mouse IL-6 ELISA MAX™ Standard kit according to the manufacturer's instructions. Plasma samples were diluted 1:4 in PBS supplemented with 0,05% Tween 20 prior to incubation.

3.7.3 Histopathology

Histological analysis of skin samples was used to determine the distribution of bacteria at the site of infection and to investigate the degree of tissue damage. All histological examinations were kindly performed by Dr. Marina Pils (Mouse pathology, Animal Experimental Unit, HZI). Skin samples obtained from uninfected and *S. pyogenes*-infected mice were fixed in 4% neutrally buffered formaldehyde and embedded in paraffin according to standard histological procedures. Sections of 3 µm thickness were stained with hematoxylin-eosin (HE) and evaluated by light microscopy blinded to the experimental groups. Inflammation was graded in the different parts of the skin (dermis, panniculus, subcutis) from 0 = no inflammation, 1 = few inflammatory infiltrates, 2 = moderate, clearly visible inflammatory infiltrates, 3 = severe inflammatory infiltrates with tissue destruction and 4 = for extreme inflammation leading to tissue destruction and causing necrosis.

3.8 Microscopy techniques

3.8.1 Immunofluorescence microscopy

Immunofluorescence microscopy was used to verify the heterologous expression of streptococcal M1 protein and chimeric M protein variants on the surface of *L. lactis*.

L. lactis strains were cultured overnight as described above and 250 to 500 µl overnight culture were centrifuged for 4 min at 5 000 rpm. The resulting bacterial pellet was incubated with the primary antibody against the streptococcal M1 protein diluted 1:100 in 50µl PBS with 10% (V/V) FCS for 45 min at 37°C. Bacterial cells were washed twice with 150 µl PBS, the bacterial pellet was resuspended in 50 µl PBS with 10% (V/V) FCS containing the secondary anti-rabbit antibody conjugated with Alexa 488 (dilution 1:200) and incubated for 45 min at 37°C with occasionally mixing. Bacteria were washed three times with 150 µl PBS and fixed in 50 µl 4% paraformaldehyde (PFA). Cell nuclei were stained by mixing 3 µl of the sample with 1 µl Fluoroshield™ with DAPI on a microscope slide. Cover slips were then placed on

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the microscope slide and samples were dried in a “wet-chamber” overnight at 4°C or 1 h at RT in the dark. Samples were analyzed using the fluorescence microscope Zeiss Axio Imager A2.

3.8.2 Double immune fluorescence microscopy

Double immune fluorescence microscopy analysis was kindly performed by Bhavya Chakrakodi and Dr. Linda Johansson from the Center for Infectious Medicine, Department of Medicine, Karolinska Institute, Karolinska University Hospital Huddinge (Stockholm, Sweden). For fluorescence microscopy skin samples obtained from uninfected and *S. pyogenes* infected mice were snap-frozen in liquid nitrogen. The biopsies were then cryosectioned to 8 µm, fixed in 2% formaldehyde and immunofluorescent stainings of serial sections were conducted as described previously (Thulin *et al.*, 2006). For immunofluorescence staining the following antibodies were used: rabbit polyclonal anti-factor XIIIa and goat polyclonal anti-*Streptococcus pyogenes* group A as well as Alexa 488 conjugated donkey-anti-rabbit IgG and Alexa 647 conjugated donkey-anti-goat IgG. Slides were mounted using DAPI supplemented mounting media. The stained sections were examined using a Nikon A1R confocal microscope.

4 RESULTS

4.1 Role of a FXIII-mediated innate immune mechanism in host defense against *S. pyogenes*

Recent studies have provided evidence supporting a role for the coagulation system in the innate immune response against *S. pyogenes*. Experimental results demonstrated that the contact system is activated on the surface of pathogenic bacteria including *S. pyogenes*, *E. coli* and *S. aureus* through specific interactions of contact factors with bacterial surface proteins. The initial contact activation induces the entire clotting cascade and leads to the immobilization of these bacteria within the newly formed fibrin networks (Herwald *et al.*, 2003; Wang *et al.*, 2010; Loof *et al.*, 2011b). The role of this innate immune mechanism in the host defense against *S. pyogenes* and in limitation of systemic bacterial spreading has been investigated within the context of this study.

4.1.1 Bacterial entrapment within fibrin clots is not effective against all *Streptococcus* species

To determine the capacity of the fibrin network to entrap different bacterial species belonging to the genus *Streptococcus* including *S. pyogenes* of serotype M1, SDSE of serotype stG11 as well as *S. oralis* and *S. pneumoniae*, *in vitro* clotting experiments were performed. Bacteria were adjusted to a transmission at 600 nm (T_{600}) of 10% representing approximately 5×10^8 CFU/ml and were further diluted in sodium citrate buffer. Finally, approximately 5×10^5 CFU (\log_{10} 5.7) bacteria were used per *in vitro* clotting reaction (inoculum, indicated as “w/o plasma” in Figure 4.1). Bacteria were mixed with normal human plasma and clotting was induced by the addition of thrombin. After fibrin network formation, clots were covered with 1% of normal human plasma. The supernatant, which contains the bacteria that are not associated with the fibrin network, was plated and the amount of immobilized bacteria was calculated by comparing the amount of bacteria in the supernatant with the amount of bacteria originally inoculated in these experiments.

The results shown in Figure 4.1 demonstrated that *S. pyogenes* M1 and SDSE but not *S. oralis* or *S. pneumoniae* were entrapped within fibrin networks. Thus, whereas the amount of *S. pyogenes* and SDSE was significantly reduced in the clot supernatant compared to the amount of originally inoculated bacteria, no differences were observed between the amount of *S. oralis* and *S. pneumoniae* in the clot supernatant and the amount of bacteria in the original inoculum (Figure 4.1). These observations lead to the conclusion that neither *S. oralis* nor *S. pneumoniae* were entrapped in fibrin clots.

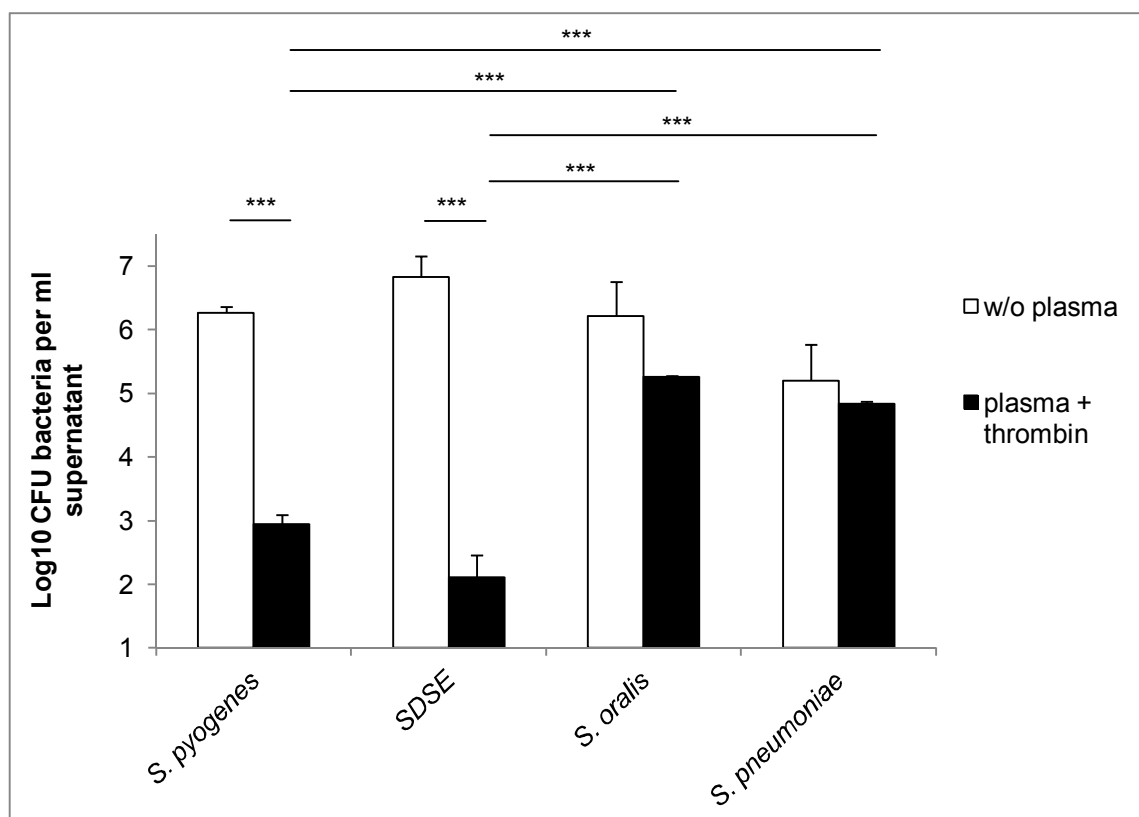


Figure 4.1: Entrapment of different species belonging to the genus *Streptococcus* within fibrin clots.

S. pyogenes M1, SDSE as well as *S. oralis* and *S. pneumoniae* were investigated for their initial interactions with the fibrin network by *in vitro* clotting assays using normal human plasma. Bacterial loads detected in the clot supernatant (black bars) were compared to bacterial loads that were originally inoculated in these assays (white bars). The graphs represent the mean \pm SD of at least three independent experiments. Statistical significance was calculated by ANOVA, *** $p < 0,001$.

The results presented in Figure 4.1 clearly demonstrated the effective entrapment of *S. pyogenes* and SDSE during *in vitro* clotting. Since previous studies reported that bacterial cross-linking within fibrin networks is mediated by interactions between bacterial surface structures and coagulation factors, the role of the major surface proteins of *S. pyogenes* and SDSE in bacterial entrapment was investigated. For this purpose, the initial interactions of *S. pyogenes* and SDSE wild type and corresponding mutant strains lacking their surface proteins M1 and stG11 (also known as FOG), respectively, were analyzed. The immobilization of the *S. pyogenes* and SDSE mutant strains was significantly reduced when compared with their corresponding wild type strains indicating a role of these surface proteins during the entrapment process (Figure 4.2 and 4.3). However, by comparing the bacterial CFU in the clot supernatant with that in the inoculum, the entrapment of these mutant strains was not completely abolished. These observations lead to the suggestion that

also other surface structures of *S. pyogenes* and SDSE influence their interactions with the fibrin network.

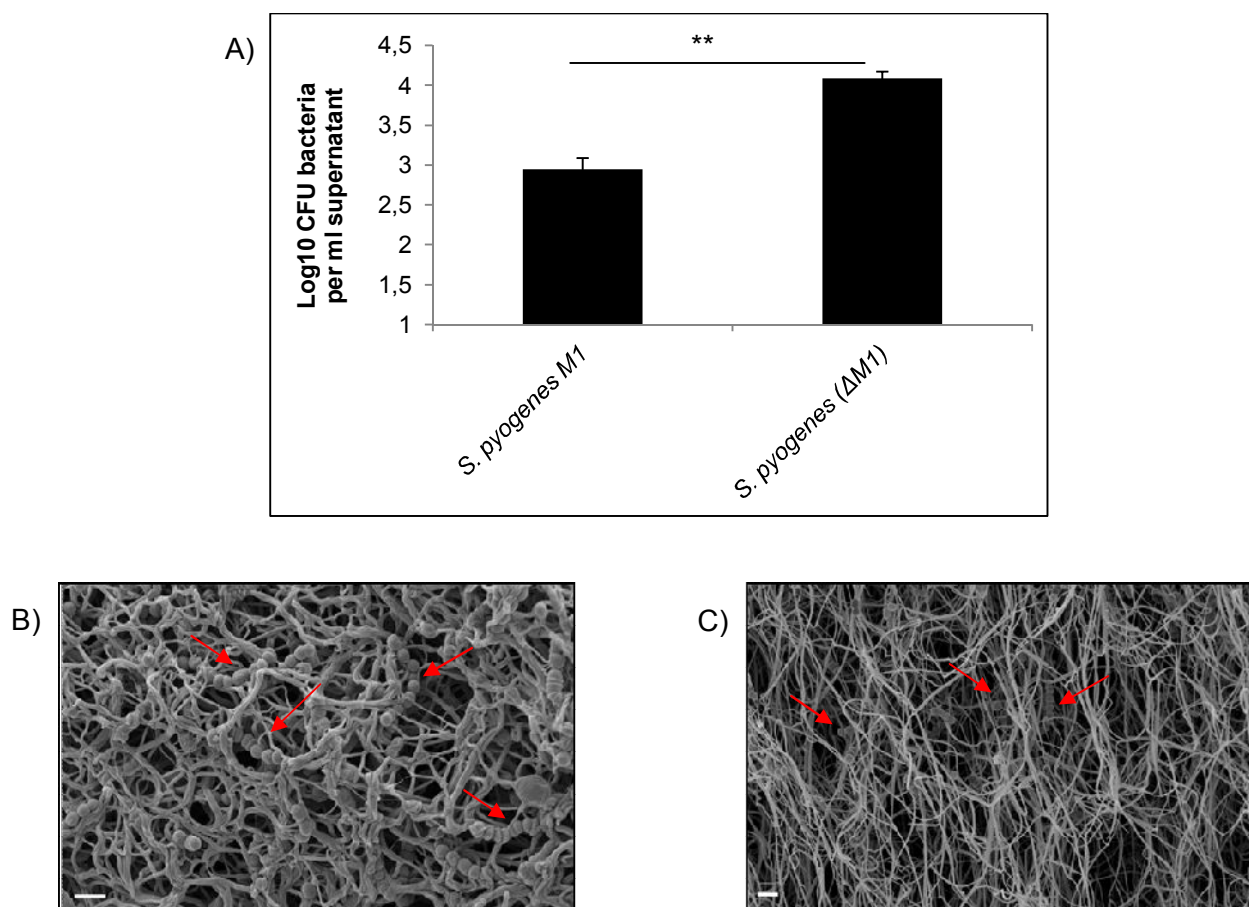


Figure 4.2: Contribution of M1 protein to the entrapment of *S. pyogenes* of serotype M1.

S. pyogenes M1 wild type and the corresponding mutant strain lacking the M1 protein (*S. pyogenes* $\Delta M1$) were investigated for their initial interactions with the fibrin network by *in vitro* clotting assays using normal human plasma. (A) Bacteria CFU in the clot supernatants of *S. pyogenes* wild type and $\Delta M1$. The graphs represent the mean \pm SD of at least three independent experiments. Statistical significance was calculated by T test: ** $p < 0.01$.

Scanning electron micrographs of the immobilization of *S. pyogenes* M1 wild type (B) and *S. pyogenes* $\Delta M1$ (C) within fibrin clots. Bacteria are indicated by red arrows.

The M1 protein-dependent entrapment of *S. pyogenes* M1 and the FOG protein-dependent entrapment of SDSE stG11 were confirmed by scanning electron microscopy. The photographs depicted in Figure 4.2 B and Figure 4.3 B show the efficient immobilization of *S. pyogenes* M1 and SDSE wild type bacteria within the fibrin network represented by tightly enwoven bacteria. The entrapment of *S. pyogenes* and SDSE mutant strains lacking the surface M1 (Figure 4.2 C) or the FOG (Figure 4.3 C) protein is strongly reduced in

comparison to the wild type strains, supporting the suggestion of a contribution of these surface proteins during clot entrapment.

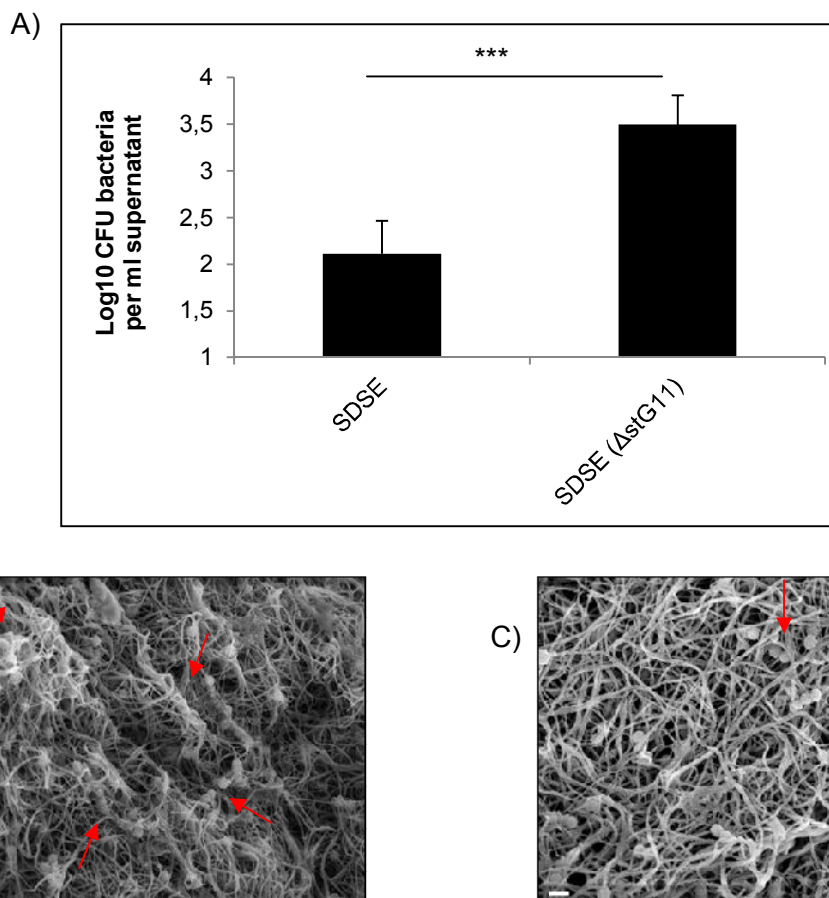


Figure 4.3: Contribution of M-like protein stG11 (FOG protein) to the entrapment of SDSE.

SDSE and the corresponding mutant strain lacking the FOG protein (SDSE Δ stG11) were investigated for their initial interactions with the fibrin network by *in vitro* clotting assays using normal human plasma. (A) Bacteria CFU in the clot supernatants of SDSE wild type and SDSE Δ stG11. The graphs represent the mean \pm SD of at least three independent experiments. Statistical significance was calculated by T test: *** $p < 0,001$. Scanning electron micrographs of the immobilization of SDSE wild type (B) and SDSE Δ stG11 (C) within fibrin clots, respectively. Bacteria are indicated by red arrows.

Taken together, the results of the *in vitro* clotting assays and scanning electron microscopy analysis using different species of the genus *Streptococcus* suggest that the bacterial entrapment is limited to certain species such as *S. pyogenes* and SDSE and that bacterial immobilization within fibrin clots seems to be largely mediated by M or M-like proteins in these bacterial species.

4.1.2 The role of FXIII in the entrapment of streptococci within fibrin networks is limited to *S. pyogenes* of the M1 serotype

FXIII was previously identified as the key component of the coagulation cascade that mediates the immobilization of different pathogenic bacteria, including *S. pyogenes*, within fibrin clots (Wang *et al.*, 2010; Loof *et al.*, 2011b). By interacting with streptococcal surface proteins such as the M1 protein, activated FXIII (FXIIIa) mediates the entrapment of *S. pyogenes* (Loof *et al.*, 2011b).

To investigate the role of FXIII in the entrapment of streptococci within the fibrin clot, *in vitro* clotting assays using *S. pyogenes* bacteria of different M serotypes as well as a SDSE strain were performed using normal and FXIII-deficient plasma. For these assays, bacteria were adjusted and diluted as mentioned before. Thus, the mean value of the bacteria inoculated in these assay was $6,12 \pm 0,43 \log_{10}$ CFU. While the entrapment of *S. pyogenes* of serotype M1 was significantly influenced by FXIII, the immobilization of *S. pyogenes* of serotypes M1-2, M3, M6, M49 and M53 as well as SDSE was not significantly influenced by FXIII (Figure 4.4). Although the bacterial CFU of *S. pyogenes* M1-2, M3, M6, M49 and M53 as well as SDSE within the supernatant of FXIII-deficient plasma clots was slightly increased compared to normal plasma, the difference did not reach statistic significance. These data indicate that the contribution of FXIII to the entrapment within the fibrin network is limited to *S. pyogenes* of M1 serotype (Figure 4.4).

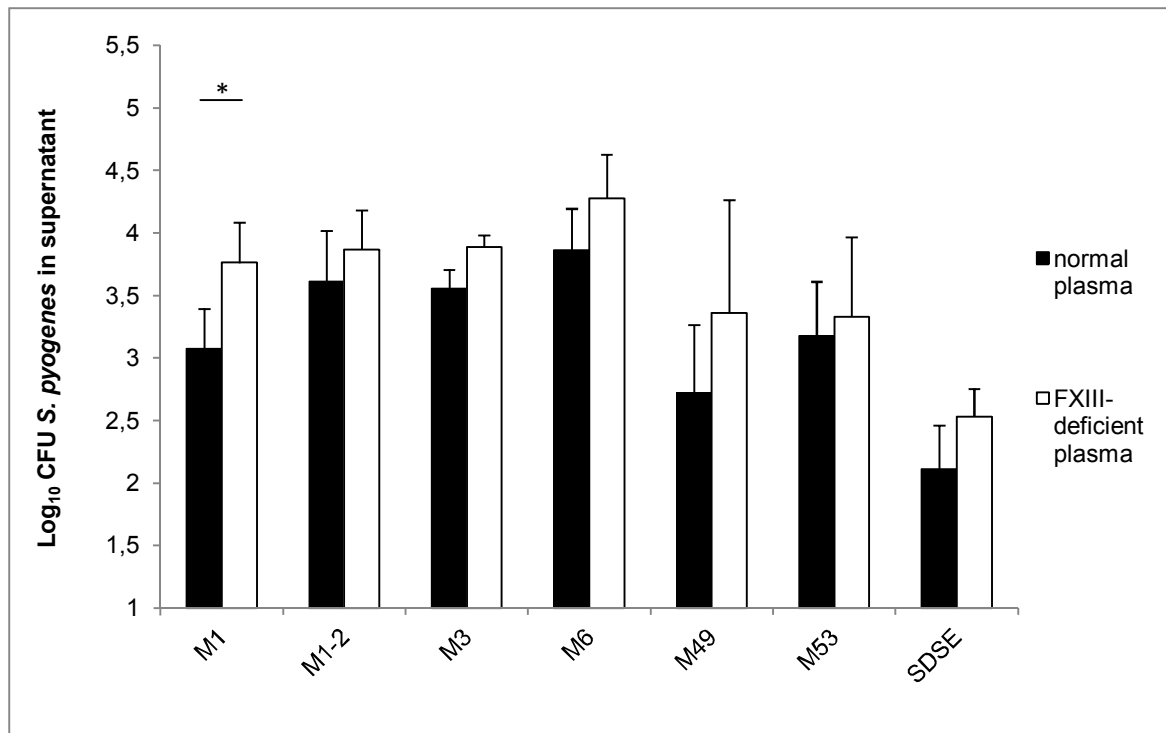


Figure 4.4: Entrapment of *S. pyogenes* of different M serotypes in the presence or absence of FXIII.

Bacterial CFU in the clot supernatant of different *S. pyogenes* M serotype strains during *in vitro* clotting using normal (black bars) and FXIII-deficient human plasma (white bars). The graphs represent the mean \pm SD of at least three independent experiments. Statistical significance was calculated by T test: * $p < 0.05$.

The influence of FXIII on the entrapment of *S. pyogenes* bacteria of the serotype M1 was also confirmed by scanning electron microscopy using the *S. pyogenes* strain AP1 (Figure 4.5). Large amounts of *S. pyogenes* AP1 bacteria were found tightly enwoven within plasma clots generated from normal plasma (Figure 4.4 A). In contrast, only single bacteria were loosely associated with the fibrin network generated from FXIII-deficient plasma as shown in Figure 4.5 B.

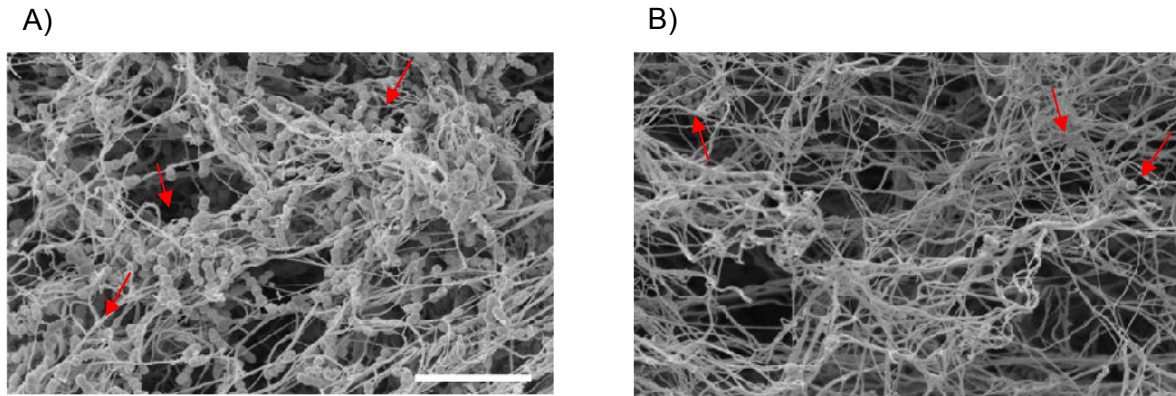


Figure 4.5: Entrapment of *S. pyogenes* bacteria of serotype M1 is influenced by FXIII.

Scanning electron micrographs showing the immobilization of *S. pyogenes* M1 strain AP1 within fibrin networks generated from normal (A) and FXIII-deficient (B) human plasma (photos taken from Loof, Deicke and Medina, 2014). Bacteria are indicated by red arrows.

To further confirm the relevance of FXIII in the fibrin-mediated immobilization of *S. pyogenes* M1 bacteria, the entrapment of different wild type *S. pyogenes* M1 strains (AP1 and 5448) and their corresponding M1-deficient isogenic mutant strains was investigated in the presence and absence of FXIII. The mean value of the bacteria inoculated in these assays was $6,11 \pm 0,3 \log_{10}$ CFU. Significant higher amounts of AP1 and 5448 bacteria were detected in the supernatant of FXIII-deficient human plasma when compared to normal human plasma (Figure 4.6). These results confirmed the less effective entrapment of *S. pyogenes* M1 bacteria in the absence of FXIII. Moreover, it was noted that the amount of bacteria in the supernatant was also significantly higher when the entrapment of the M1-deficient mutant strains was compared to that of the wild type strains in normal plasma (Figure 4.6). If both, the streptococcal M1 protein and FXIII are missing nearly the whole bacterial inoculum used for the assay, was detected in the supernatant indicating that the bacterial entrapment was nearly but not completely abolished without FXIII and the M1 protein (Figure 4.6). These results demonstrated the relevance of FXIII and the M1 protein for fibrin immobilization of *S. pyogenes* but also indicate the contribution of additional factors in the entrapment process.

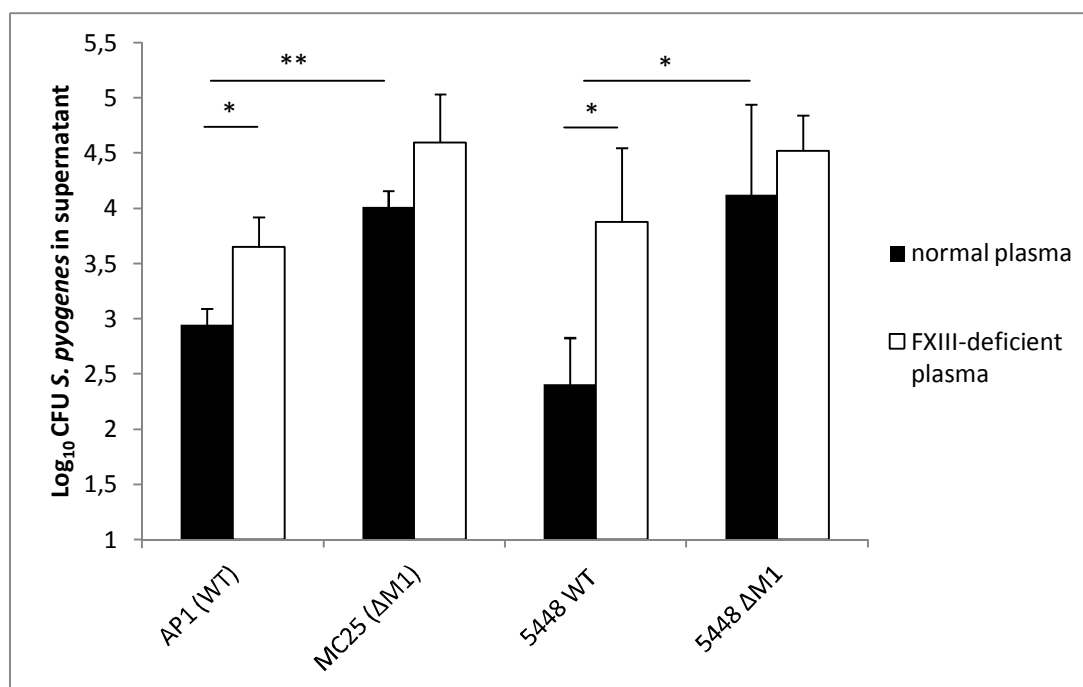


Figure 4.6: Entrapment of *S. pyogenes* of serotype M1 within fibrin clots is influenced by FXIII and streptococcal M1 protein.

Bacterial CFU in the clot supernatant of different *S. pyogenes* M1 serotype strains during *in vitro* clotting. Amounts of *S. pyogenes* AP1 and 5448 wild type bacteria as well as their corresponding M1-deficient mutant strains in the presence of normal (black bars) or FXIII-deficient (white bars) plasma are shown. The graphs represent the mean \pm SD of at least three independent experiments. Statistical significance was calculated by T test: * $p < 0.05$ and ** $p < 0.01$.

4.2 Molecular mechanism underlying the FXIII-mediated entrapment of *S. pyogenes* M1 bacteria within the fibrin network

Published data (Loof *et al.*, 2011b) as well as experiments performed within the context of this thesis demonstrated a contribution of FXIII to the immobilization of *S. pyogenes* M1 bacteria within fibrin clots. As an interaction between FXIII and the streptococcal M1 protein was previously shown using immunofluorescence microscopy and transmission electron microscopy (Loof *et al.*, 2011b), the possibility of a covalent linkage between the streptococcal M1 protein and FXIII was investigated in this study. For this purpose, fibrinogen conversion assays were established by mixing purified fibrinogen, thrombin and FXIII in different reactions and incubating them for 2 h at 37°C to allow fibrin formation. The conversion of fibrinogen to fibrin in this assay was determined by SDS PAGE and fibrin(ogen) western blot analysis.

RESULTS

Denatured fibrinogen, separated by SDS PAGE, is characterized by three protein bands of approximately 65 kDa, 52 kDa and 46 kDa representing the α -, β - and γ -chains of fibrinogen, respectively (Figure 4.6; Weisel, 2005). The addition of thrombin to fibrinogen mediates the conversion to fibrin that can be followed by the formation of a protein band of approximately 95 kDa representing the fibrin γ - γ dimers (Figure 4.7). The incubation of fibrinogen with FXIII did not alter the band pattern of fibrinogen, while incubation of fibrinogen with thrombin and FXIII resulted in cross-linked fibrin polymers characterized by the generation of γ - γ dimer bands and high molecular α polymer bands (Figure 4.7). The sample containing thrombin and FXIII served as a control. The results of these first experiments illustrated that this assay was suitable to analyze fibrinogen conversion *in vitro*.

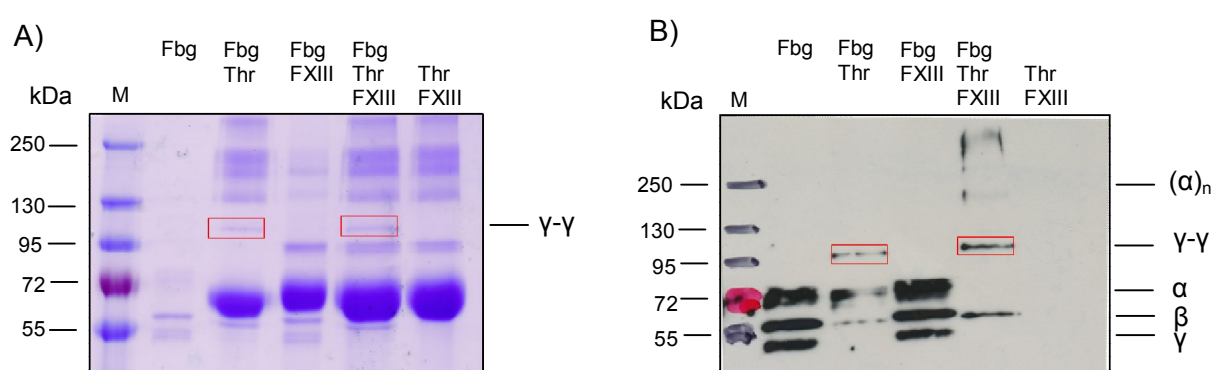


Figure 4.7: *In vitro* conversion of fibrinogen to fibrin.

The conversion of fibrinogen to fibrin *in vitro* was analyzed by SDS PAGE (A) and western blot against fibrin(ogen) (B). *PageRuler Prestained Protein Ladder* was used as a molecular weight standard (M). Protein samples were diluted in a 1:10 ratio with 1 x SDS sample buffer for the western blot analysis. Fbg: fibrinogen; Thr: thrombin; FXIII: factor XIII; γ - γ : fibrin γ - γ dimers; $(\alpha)_n$: fibrin α polymers.

4.2.1 Streptococcal M1 protein inhibits the fibrinogen conversion to fibrin

To analyze the interactions between the streptococcal M1 protein with fibrinogen and FXIII, fibrinogen conversion assays were performed in the presence of recombinant streptococcal M1 protein (Figure 4.8). In contrast to the hypothesis, no covalent complexes between the M1 protein and FXIII were observed by SDS PAGE and fibrin(ogen) blot after *in vitro* clotting reaction. Thus, no γ - γ dimers were observed after incubation of fibrinogen with thrombin in the presence of 0.5 μ g as well as 5 μ g of M1 protein, indicating that the conversion to fibrin was inhibited (Figure 4.8). However, when FXIII was added to fibrinogen, thrombin and M1 protein, fibrin γ - γ dimers as well as fibrin α polymers were detected again (Figure 4.8).

RESULTS

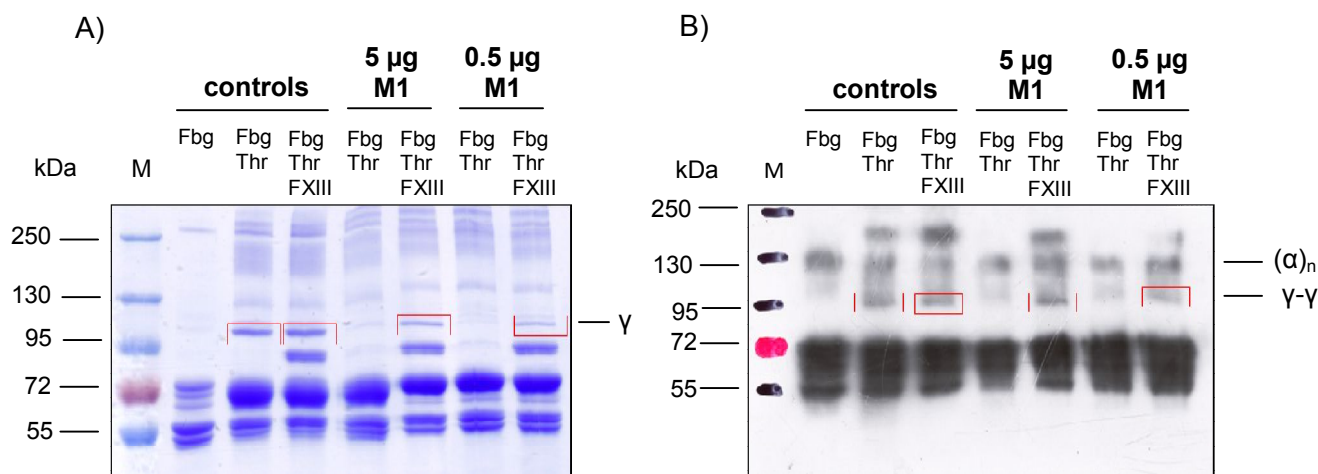


Figure 4.8: Fibrinogen conversion *in vitro* in the presence of streptococcal M1 protein.

The conversion of fibrinogen to fibrin *in vitro* in the presence of 0.5 and 5 µg of streptococcal M1 protein was analyzed by SDS PAGE and Coomassie staining (A) as well as by western blot analysis using anti-fibrin(ogen) antibody (B). *PageRuler Prestained Protein Ladder* was used as a molecular weight standard (M). Protein samples were diluted in a 1:10 ratio with 1 x SDS sample buffer for the western blot analysis. Fbg: fibrinogen; Thr: thrombin; FXIII: factor XIII; M1: streptococcal M1 protein; γ-γ: fibrin γ-γ dimers; (α)_n: fibrin α polymers.

The results of these experiments lead to the interesting observation that the M1 protein was able to inhibit the fibrinogen conversion to fibrin during *in vitro* clotting. However, addition of FXIII was able to revert the inhibitory effect of M1 protein and fibrin was generated. These data suggest that FXIII could counteract the inhibitory effects of the M1 protein during fibrin formation.

To clarify if the inhibitory effect of the fibrin formation was an exclusive feature of the M1 protein or if it is also mediated by other M proteins, fibrinogen conversion assays were performed in the presence of recombinant streptococcal M1-2, M3 and M55 protein as well as the M-like protein FOG of SDSE (Figure 4.9). The M1-2, M3, M55 and the FOG proteins failed to inhibit fibrinogen conversion in the absence and in the presence of FXIII as indicated by the formation of γ-γ dimers as well as fibrin α polymers (Figure 4.9). These results indicate that only the streptococcal M1 protein was able to inhibit the fibrin formation. However, this inhibitory effect was reverted in the presence of FXIII.

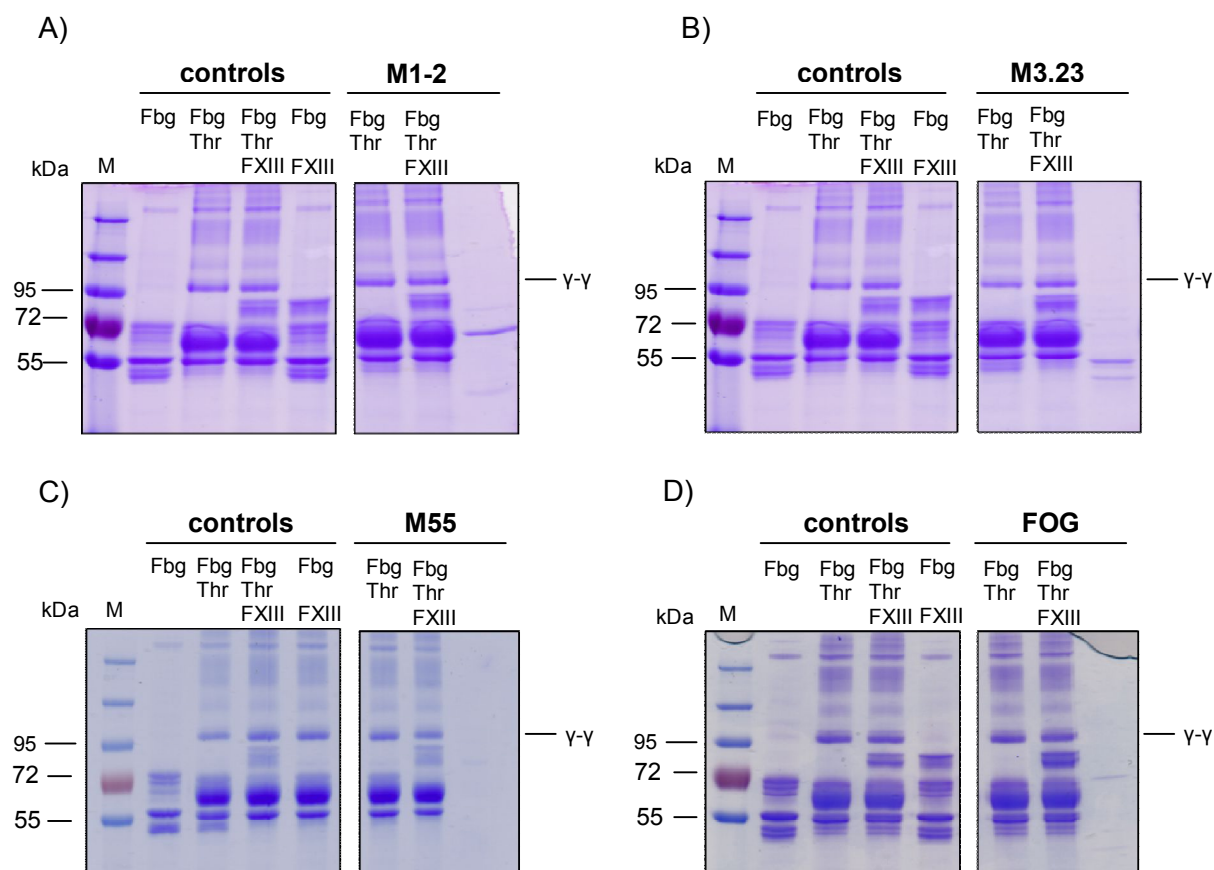


Figure 4.9: Fibrinogen conversion *in vitro* in the presence of different streptococcal M and M-like proteins.

The conversion of fibrinogen to fibrin *in vitro* in the presence of 5 μ g streptococcal M1-2 (A), M3.23 (B), M55 (C) and FOG (D) protein was analyzed by SDS PAGE and Coomassie staining. *PageRuler Prestained Protein Ladder* was used as a molecular weight standard (M). Fbg: fibrinogen; Thr: thrombin; FXIII: factor XIII; M1: streptococcal M1 protein; γ - γ : fibrin γ - γ dimers.

4.2.2 The hypervariable A region within the streptococcal M1 protein is responsible for the inhibition of fibrinogen conversion

To further delineate the region of the M1 protein responsible for the inhibitory effect, different constructs containing different domains of the M1 protein were generated and used in fibrinogen conversion experiments (Figure 4.10)

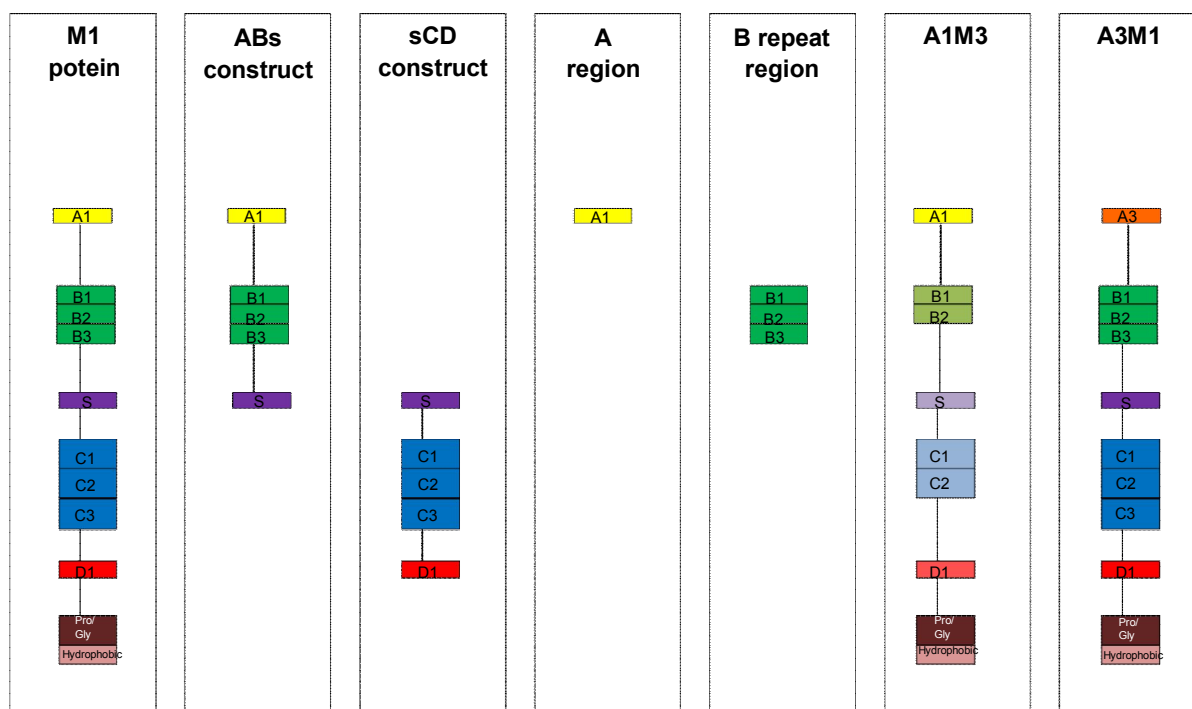


Figure 4.10: Overview of the M1 protein constructs and chimeric M proteins.

Schematic representation of the constructs containing the different M1 protein domains as well as the chimeric M proteins A1M3 and A3M1. Yellow: A1 region; green: B repeat region; violet: S region; blue: C repeat region; red: D repeat region; orange: A3 region.

M1 protein constructs containing the N terminal domains (the ABs construct) and the C terminal domains (the sCD construct) were compared with the full-length M1 protein for their capacity to inhibit fibrinogen conversion (Figure 4.11). Whereas the ABs construct containing the fibrinogen-binding regions exhibited a similar capacity as the full-length M1 protein to inhibit the fibrin formation in the absence of FXIII, the sCD construct did not affect fibrin formation (Figure 4.11). Thus, using this experimental approach, the N terminal part of the M1 protein was identified as responsible for the inhibition of fibrin formation.

To further identify the domain within the N terminal part of the M1 protein responsible for the inhibitory effect, constructs containing either the A region or the B repeat region of the M1 protein were tested in fibrinogen conversion assays (Figure 4.11). Interestingly, the B repeat region, which represents the classical fibrinogen binding domain within the M1 protein, was incapable to inhibit the fibrinogen conversion to fibrin, neither in the presence nor in the absence of FXIII (Figure 4.11). However, the small A region of the streptococcal M1 protein alone was able to inhibit fibrin formation, but only in the absence of FXIII (Figure 4.10).

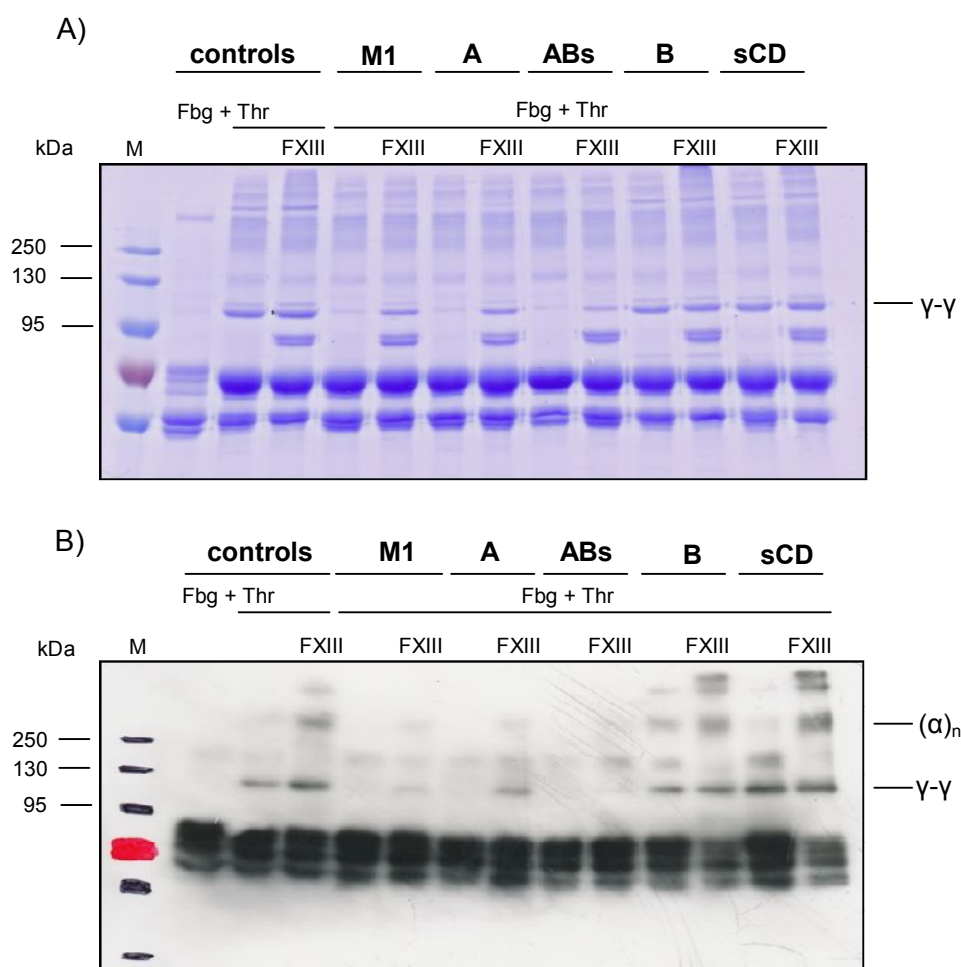


Figure 4.11: Capacity of the different M1 protein domains to inhibit fibrinogen conversion.

The conversion of fibrinogen to fibrin *in vitro* in the presence of 5 μ g streptococcal M1 protein constructs was analyzed by SDS PAGE and Coomassie staining (A) as well as western blot analysis using anti-fibrin(ogen) antibody (B). *PageRuler Prestained Protein Ladder* was used as a molecular weight standard (M). Protein samples were diluted in a ratio 1:10 with 1 x SDS sample buffer for western blot analysis. Fbg: fibrinogen; Thr: thrombin; FXIII: factor XIII; M1: streptococcal M1 protein; A: M protein A region; B: M protein B repeat region; ABs: M protein construct containing the N terminal A, B and S region; sCD: M protein construct containing the C terminal S, C and D region; γ - γ : fibrin γ - γ dimers; (α)_n: fibrin α polymers.

To confirm the results described above, fibrinogen conversion experiments were performed in the presence of recombinant chimeric M proteins as a proof of concept. To generate these chimeras, the A regions of the M1 and the M3 protein were exchanged resulting in the chimeric proteins A1M3, representing a M3 protein containing the A region of the M1 protein, and A3M1, representing a M1 protein containing the A region of the M3 protein. As shown in Figure 4.12, the A1M3 but not the A3M1 protein was capable to inhibit the fibrin formation in the absence of FXIII. The ability of the M1 protein to inhibit the fibrin formation in the absence

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of FXIII was lost if only the A region was exchanged against the A region of the M3 protein. However, fibrinogen conversion was detected in the presence of the A1M3 protein after addition of FXIII (Figure 4.12).

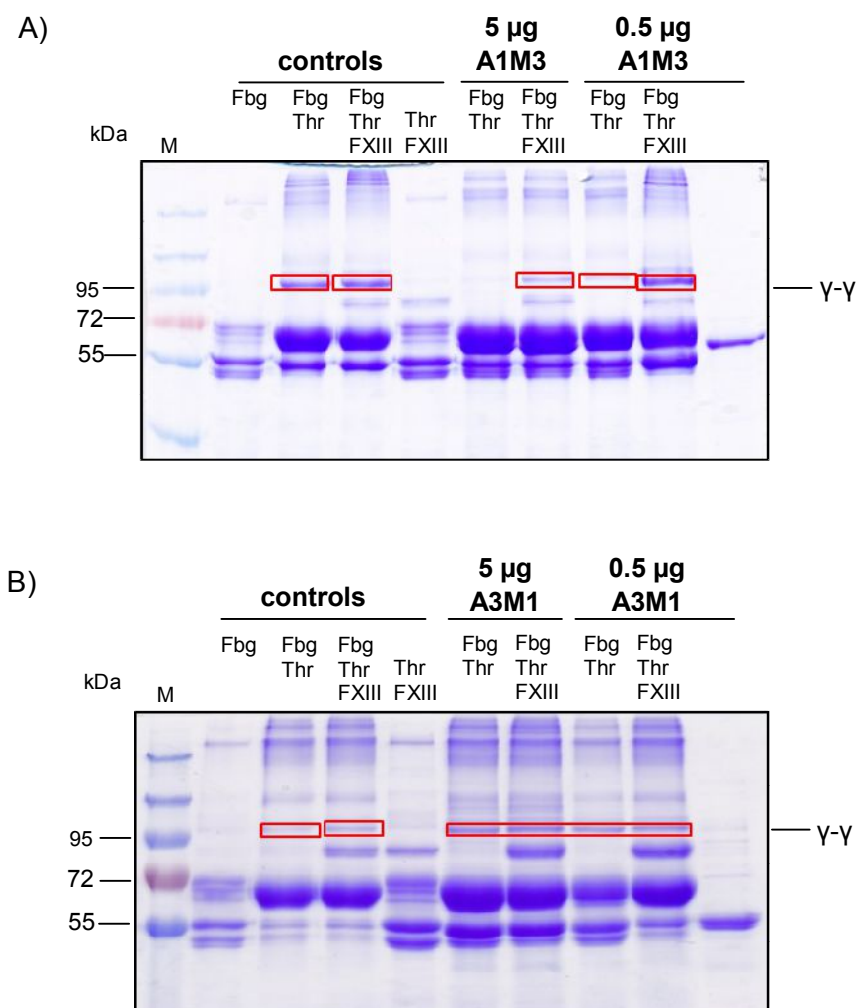


Figure 4.12: Fibrinogen conversion *in vitro* in the presence of the chimeric streptococcal M protein constructs A1M3 and A3M1.

The conversion of fibrinogen to fibrin *in vitro* in the presence of 0.5 and 5 µg A1M3 (A) and A3M1 (B) M protein constructs was analyzed by SDS PAGE and Coomassie staining. As a molecular weight standard (M) the *PageRuler Prestained Protein Ladder* was used. Fbg: fibrinogen; Thr: thrombin; FXIII: factor XIII; M1: streptococcal M1 protein; γ-γ: fibrin γ-γ dimers.

4.2.3 The A region of the streptococcal M1 protein binds fibrinogen

The ability of streptococcal M proteins to bind fibrinogen was already described in 1933 by Tillet and Garner (Tillett and Garner, 1933). In 2000, the M protein B repeat region was identified as the fibrinogen-binding domain within the M protein (Ringdahl *et al.*, 2000). Interestingly, the results described above demonstrate that the A region of the streptococcal

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M1 protein was responsible for the inhibition of the fibrinogen conversion. These observations lead to the suggestion that fibrinogen-binding is perhaps a precondition for the inhibition of the fibrin formation. Therefore, the fibrinogen binding capacity of the A region of the M1 protein was investigated in fibrinogen pulldown experiments (Figure 4.13).

To this end, different constructs representing the different domains of the M1 protein were covalently coupled to magnetic beads followed by incubation with fibrinogen as described in section 3.4.5. Fibrinogen bound to magnetic beads coated with either the full-length M1 protein, the A region or the B repeat region was eluted and visualized by SDS-PAGE (Figure 4.13). The results revealed that, besides the classical fibrinogen-binding B repeat region, also the A region has the capacity to bind fibrinogen. This was clearly indicated by the detection of fibrinogen within the elution fractions (Figure 4.13). The sCD construct served as a negative control (Figure 4.13). The identity of the eluted proteins was confirmed by western blot analysis against fibrin(ogen) (data not shown).

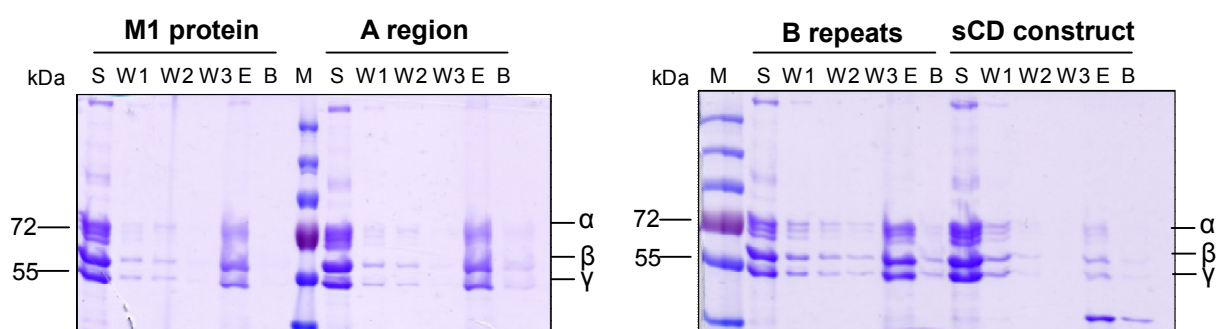


Figure 4.13: Fibrinogen binding capacity of the different M1 protein domains.

Fibrinogen pulldown assays using either the full-length M1 protein, the A region, the B repeat region or the sCD construct were analyzed by SDS PAGE and Coomassie staining. *PageRuler Prestained Protein Ladder* was used as a molecular weight standard (M). S: supernatant; W1 – 3: washing fractions; E: eluate; B: beads after elution; α , β , γ : α , β , γ chains of fibrinogen.

The results of the fibrinogen pulldown assay demonstrated the capacity of the A region of the streptococcal M1 protein to bind fibrinogen. These results are in accordance with a recent study reported by Macheboeuf and colleagues (Macheboeuf *et al.*, 2011). Hence, besides the B repeat region also the A region contributes to fibrinogen-binding of the M1 protein. These observations suggest that the fibrinogen binding capacity of the A region may be related to its ability to inhibit the fibrinogen conversion *in vitro*.

4.2.4 *S. pyogenes* of serotype M1 mediates the inhibition of fibrinogen conversion *in vitro*

The previously described data were derived from *in vitro* fibrinogen conversion experiments using recombinant M proteins and M protein constructs. Therefore, the capacity of *S. pyogenes* M1 to influence the fibrinogen conversion *in vitro* was investigated in fibrinogen conversion experiments using whole bacteria (see section 3.5.3.2).

Similar to the results with the recombinant protein, *S. pyogenes* M1 strain AP1 inhibited the conversion of fibrinogen to fibrin in the absence of FXIII (Figure 4.14). Thus, the protein band around 95 kDa representing the γ - γ dimer was absent in the SDS PAGE in the presence of *S. pyogenes* AP1 (Figure 4.14). In contrast, when *S. pyogenes* MC25, the isogenic mutant strain lacking the M1 protein was used, the *in vitro* fibrin formation was not affected and the γ - γ dimers were observed (Figure 4.14). The γ - γ dimers of fibrin were also detected in the presence of the *S. pyogenes* M3 strain A600, which accordingly did not influence the fibrin formation *in vitro* (Figure 4.14). These observations support the previous results that only the M1 protein has the ability to suppress fibrin formation. Western Blot analysis against fibrin(ogen) confirmed the results of the SDS PAGE (data not shown).

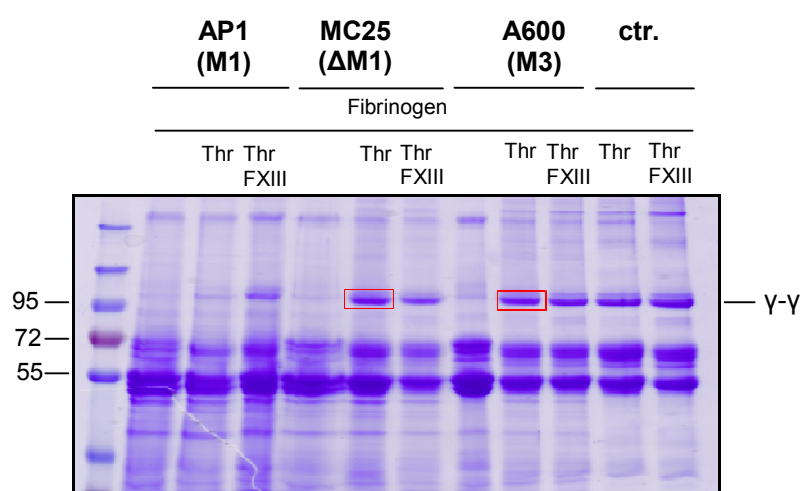


Figure 4.14: Fibrinogen conversion *in vitro* in the presence of *S. pyogenes*.

The conversion of fibrinogen to fibrin *in vitro* in the presence of *S. pyogenes* AP1 (M1), MC25 (ΔM1) and A600 (M3) was analyzed by SDS PAGE and Coomassie staining. As a molecular weight standard (M) the *PageRuler Prestained Protein Ladder* was used. Fbg: fibrinogen; Thr: thrombin; FXIII: factor XIII; γ - γ : fibrin γ - γ dimers, ctr.: controls without bacteria.

To confirm that the fibrinogen conversion is also inhibited by other *S. pyogenes* strains of serotype M1, experiments were performed using the M1 strains 90-226 and 5448 as well as their corresponding M1 deficient mutant strains (90-226 ΔM1 and 5448 ΔM1). Indeed, similar

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to the observation obtained from experiments with strain AP1 (Figure 4.14), fibrin formation was also inhibited by *S. pyogenes* M1 wild type strains 90-226 and 5448 in the absence of FXIII but not by the corresponding mutant strains lacking the M1 protein (Figure 4.15).

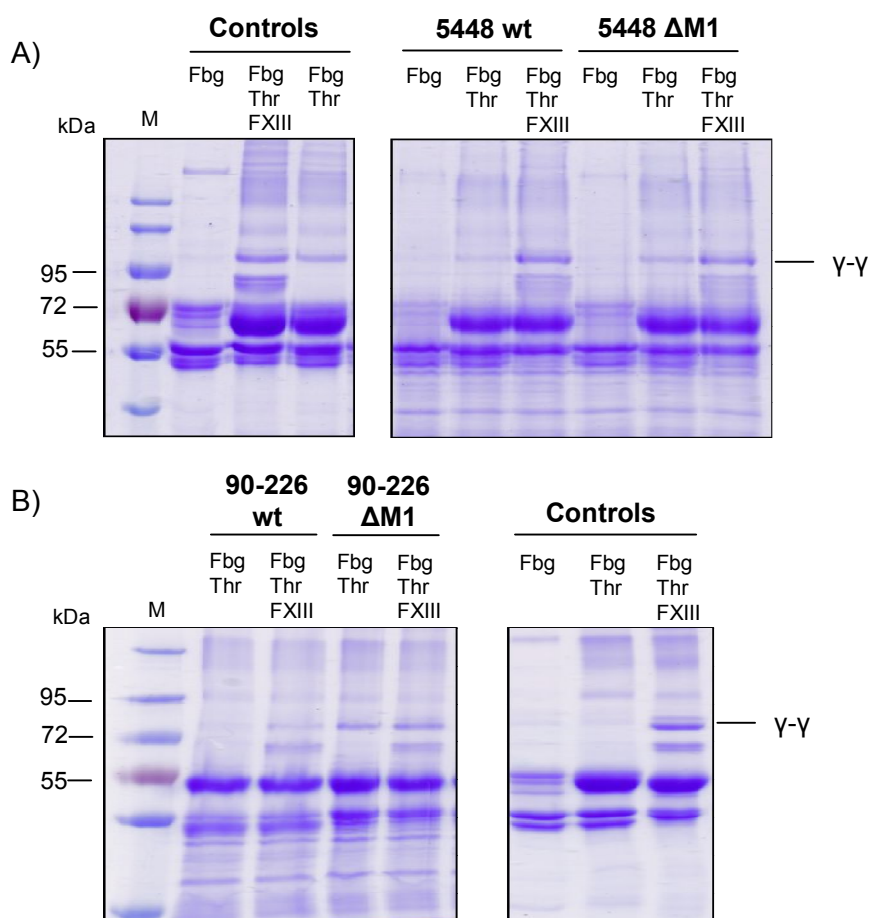


Figure 4.15: Fibrinogen conversion *in vitro* in the presence of *S. pyogenes* serotype M1.

The conversion of fibrinogen to fibrin *in vitro* in the presence of *S. pyogenes* 5448 and its corresponding M1-deficient mutant strain (5448 Δ M1) (A) as well as *S. pyogenes* 90-226 and the corresponding mutant strain 90-226 Δ M1 (B) were analyzed by SDS PAGE and Coomassie staining. *PageRuler Prestained Protein Ladder* was used as a molecular weight standard (M). Fbg: fibrinogen; Thr: thrombin; FXIII: factor XIII; γ - γ : fibrin γ - γ dimers, ctr.: controls without bacteria.

Taken together, the results of fibrinogen conversion experiments using different *S. pyogenes* strains are in good agreement with the observations from experiments using recombinant M proteins. The data suggest that M1 protein either recombinantly produced and purified or expressed on the bacterial surface is able to inhibit the fibrin formation.

4.2.5 Heterologous expressed streptococcal M1 protein on the surface of *L. lactis* inhibits fibrinogen conversion

Since in addition to the M protein, *S. pyogenes* expresses several surface-associated proteins that can interact with fibrinogen, a heterologous expression system was used to confirm the exclusivity of M1 protein in the inhibition of fibrinogen conversion. For this purpose, non-pathogenic *L. lactis* strains heterologous expressing the streptococcal M1 protein and chimeric M protein constructs A1M3 and A3M1 on their surface were generated. Because *L. lactis* bacteria do not express surface proteins capable to interact with fibrinogen, any effect on the fibrinogen conversion observed with the generated *L. lactis* strains can be solely attributed to the action of the heterologous expressed M proteins.

The expression of the M1 protein as well as the chimeric M proteins, A1M3 and A3M1, on the surface of *L. lactis* was visualized by immunofluorescence microscopy (Figure 4.16). For this purpose, *L. lactis* bacteria were prepared as described in section 3.8.1 and incubated with a specific primary antibody against the M1 protein followed by a green fluorescence-conjugated secondary antibody. Green fluorescence was only detected if *L. lactis* strains expressed the M1 protein or the chimeric proteins A1M3 and A3M1 on the bacterial surface. Thus, it was verified that the generated *L. lactis* strains expressed the M proteins mentioned above since only these strains but not the *L. lactis* wild type strain exhibited green fluorescence under microscopic examination (Figure 4.16). The cell nuclei were stained with DAPI in blue to visualize bacteria in all samples.

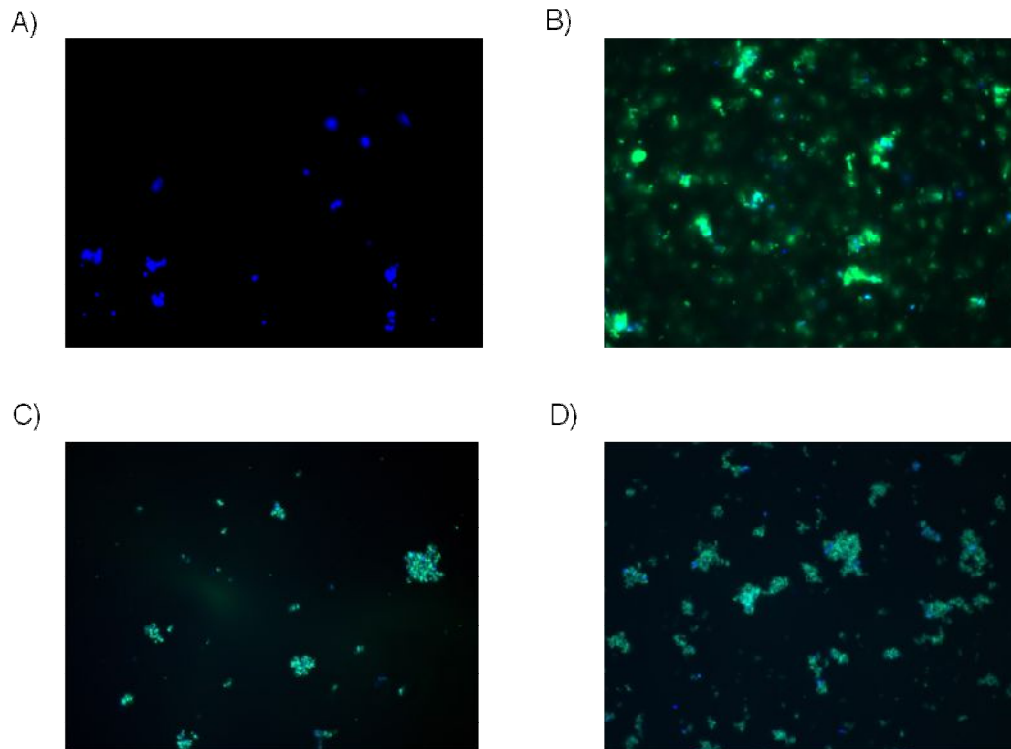


Figure 4.16: Heterologous expression of streptococcal M proteins on the surface of *L. lactis*. Immunofluorescence microscopy analysis of *L. lactis* wild type bacteria (A) and *L. lactis* strains expressing the streptococcal M1 protein (B), the A1M3 protein (C) and the A3M1 protein (D) on their surface, stained with a specific primary antibody against the M1 protein and a green fluorescence-conjugated secondary antibody (green). Cell nuclei were stained with DAPI (blue).

The *L. lactis* wild type and M protein-expressing strains were then used in fibrinogen conversion experiments. The results from the SDS PAGE and western blot analysis presented in Figure 4.17 show that while fibrin formation was not influenced by *L. lactis* wild type bacteria, *L. lactis* strain expressing the streptococcal M1 protein on its surface was capable to inhibit fibrinogen conversion in the absence of FXIII. This was demonstrated by the absence of γ - γ dimer bands in these samples (Figure 4.17). These data indicate that heterologous expressed streptococcal M1 protein is also able to inhibit the fibrin formation *in vitro*. To confirm this further, the red labeled protein bands were analyzed via mass spectrometry and identified as fibrin γ - γ dimers (data not shown).

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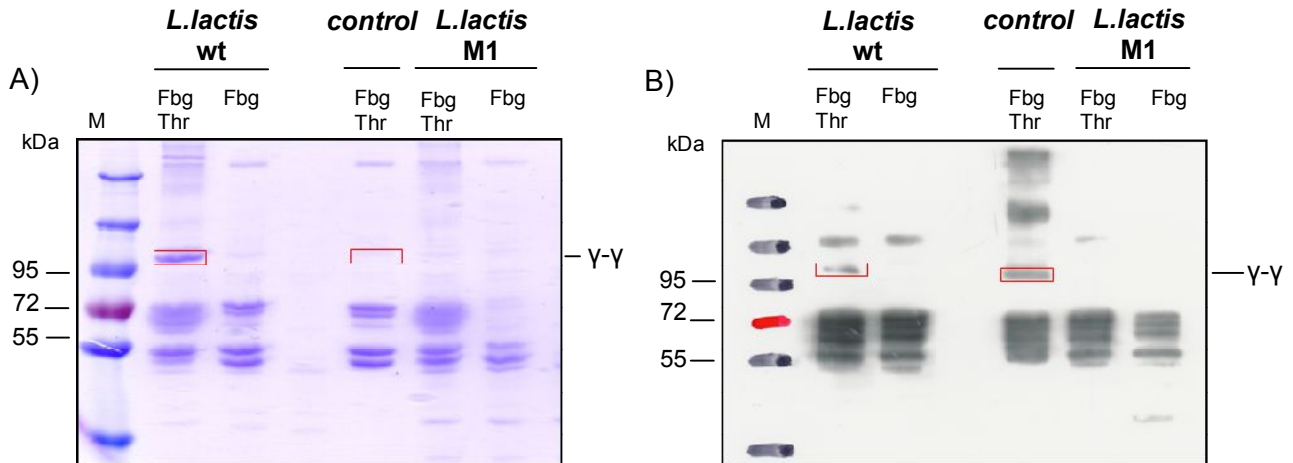


Figure 4.17: Fibrinogen conversion in the presence of *L. lactis* strains.

The conversion of fibrinogen to fibrin *in vitro* in the presence of *L. lactis* wild type and *L. lactis* expressing streptococcal M1 protein was analyzed by SDS PAGE and Coomassie staining (A) as well as western blot analysis using anti-fibrin(ogen) antibody (B). *PageRuler Prestained Protein Ladder* was used as a molecular weight standard (M). Protein samples were diluted in a ratio 1:25 with 1 x SDS sample buffer for the western blot analysis. Fbg: fibrinogen; Thr: thrombin; γ-γ: fibrin γ-γ dimers.

Furthermore, fibrinogen conversion experiments using *L. lactis* mutant strains expressing the chimeric M protein constructs confirmed the importance of the A region of the M1 protein for the inhibition of fibrinogen conversion. In the absence of FXIII, the *L. lactis* strain expressing the A1M3 protein inhibited fibrin formation (Figure 4.18). Nevertheless, in the presence of FXIII, fibrinogen was converted to fibrin as shown by the presence of the γ-γ dimer and α polymers. In contrast, the *L. lactis* strain expressing the A3M1 protein did not influence the fibrin formation neither in the presence nor in the absence of FXIII (see Figure 4. 18).

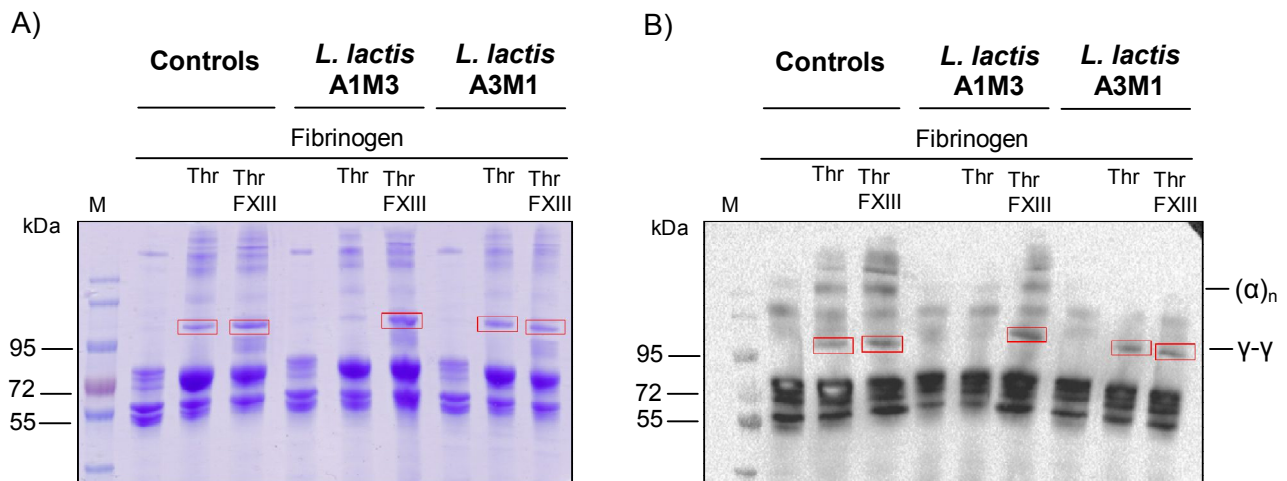


Figure 4.18: Fibrinogen conversion *in vitro* in the presence of *L. lactis* expressing A1M3 and A3M1.

The conversion of fibrinogen to fibrin *in vitro* in the presence of *L. lactis* strains expressing the A1M3 or the A3M1 protein was analyzed by SDS PAGE and Coomassie staining (A) as well as western blot analysis using anti-fibrin(ogen) antibody (B). *PageRuler Prestained Protein Ladder* was used as a molecular weight standard (M). Protein samples were diluted in a ratio 1:15 with 1 x SDS sample buffer for the western blot analysis. Fbg: fibrinogen; Thr: thrombin; FXIII: factor XIII; M1: streptococcal M1 protein; γ-γ: fibrin γ-γ dimers; (α)_n: fibrin α polymers.

In conclusion, the results obtained from the experiments using *L. lactis* bacteria suggest that the streptococcal M1 protein and the A region of this protein were able to inhibit the fibrinogen conversion to fibrin *in vitro* also when they were expressed on the surface of non-pathogenic bacteria.

4.3 Role of FXIII during *S. pyogenes* infections *in vivo*

Previous studies reported that the FXIII-mediated entrapment of *S. pyogenes* M1 bacteria within the fibrin network *in vivo* represents an early innate immune mechanism to retain invading bacteria at the site of infection. Thus, in a previously described subcutaneous *S. pyogenes* infection model leading to systemic infection and sepsis (Toppel *et al.*, 2003), mice deficient in FXIII developed more severe signs of inflammation when compared with wild type animals (Loof *et al.*, 2011b). Within the context of this PhD thesis, the impact of FXIII during skin infections caused by *S. pyogenes* of serotype M1 was investigated. To this end, the course of a *S. pyogenes* M1 skin and soft tissue infection in mice deficient in FXIII was compared with the infection in the corresponding wild type animals.

4.3.1 FXIII contributes to host defense against *S. pyogenes* at the site of infection *in vivo*

As contact activation on the surface of pathogenic bacteria leads to the induction of the entire clotting cascade including FXIII *in vitro* (Loof *et al.*, 2011b), the activation of FXIII at the site of infection was investigated *in vivo*. To this end, CBA wild type and FXIII-deficient mice were subcutaneously infected with *S. pyogenes* strain 5448 of serotype M1 and skin biopsies were prepared 24 h after bacterial inoculation for immunofluorescence microscopy analysis (Figure 4.19). Skin biopsies from the local focus of infection were snap-frozen and stained by incubation with primary antibodies recognizing *S. pyogenes* or the activated form of murine and human FXIII (FXIIIa), respectively followed by fluorescence-conjugated secondary antibodies. As demonstrated in Figure 4.19, large amounts of bacteria were detected in skin samples of all groups of mice. Additionally, a widespread positive staining for FXIIIa in the same area in which the bacteria were located was detected in wild type mice (Figure 4.19, upper panel). As expected, no fluorescence signals for FXIIIa were observed in biopsies obtained from infected FXIII-deficient mice (Figure 4.19, middle panel). However, the staining for active FXIIIa was positive after infection of FXIII-deficient mice which were reconstituted with Fibrogammin after *S. pyogenes* M1 infection (Figure 4.19, lower panel).

Consequently, these results lead to the suggestion that FXIII is activated at the site of *S. pyogenes* infection in wild type mice. The detection of FXIIIa in close proximity to *S. pyogenes* in wild type as well as in Fibrogammin-reconstituted FXIII-deficient mice indicated the participation of FXIII in the early innate immune response against *S. pyogenes*.

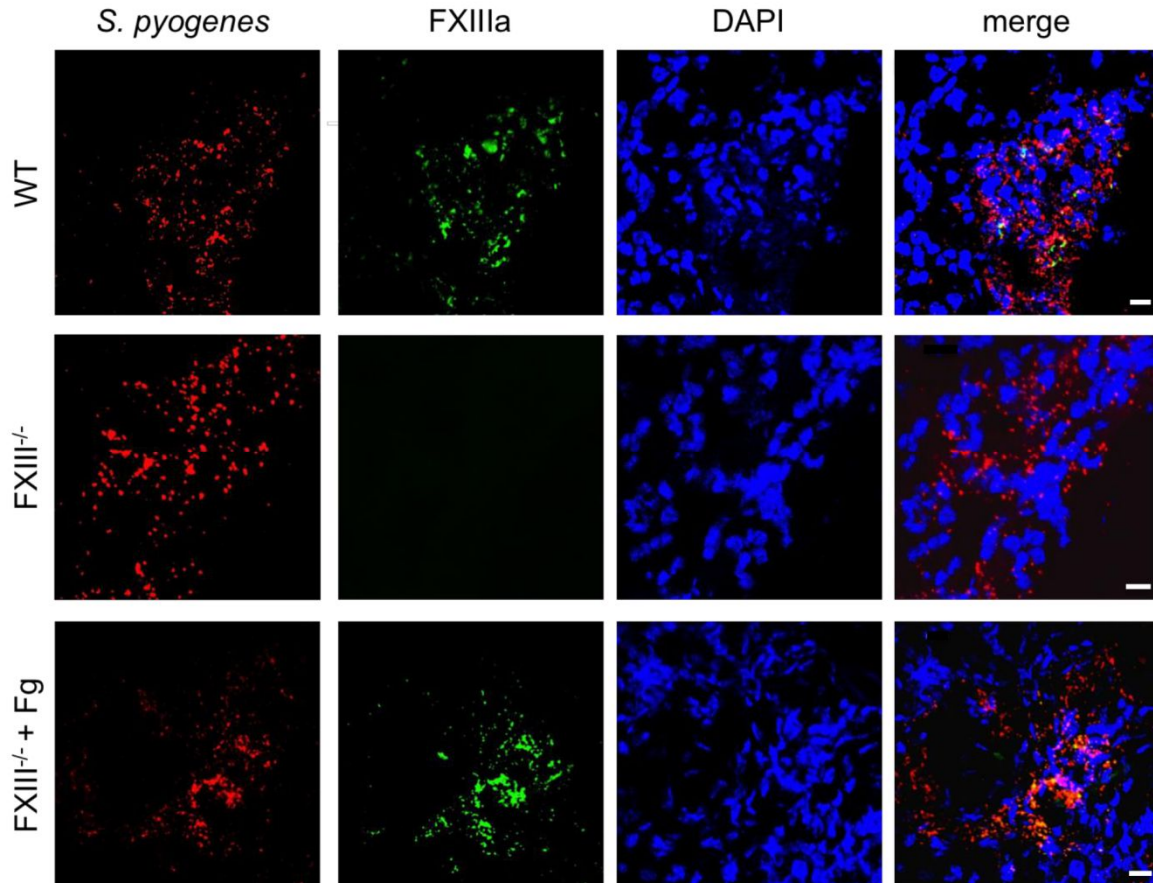


Figure 4.19: FXIII activation at the site of *S. pyogenes* infection.

Immunofluorescence microscopy analysis of skin biopsies obtained from infected wild type (upper panel), FXIII-deficient (middle panel), and FXIII-deficient mice reconstituted with Fibrogammin (Fg) (lower panel) 24h after bacterial inoculation. Samples were snap-frozen, sectioned, and stained for *S. pyogenes* (red) and activated FXIII (FXIIIa) (green). Cell nuclei were stained with DAPI (blue). The scale bars represent 10 μ m. WT: wild type mice; FXIII^{-/-}: FXIII-deficient mice.

To further investigate the role of FXIII in the host defense against *S. pyogenes* skin infections, skin biopsies obtained from infected mice at 24 h after bacterial inoculation were additionally prepared for histopathological examination using HE staining. Whereas skin samples obtained from uninfected wild type (Figure 4.20 A) or FXIII-deficient mice (Figure 4.20 B) show no signs of inflammation, samples from *S. pyogenes*-infected wild type mice exhibited moderate inflammation and mild dermatitis accompanied by edema, panniculitis and inflammation of the subcutis (Figure 4.20 C). In contrast, *S. pyogenes* caused severe necrotic dermatitis, severe panniculitis and extreme subcutaneous inflammation in FXIII-deficient mice (Figure 4.20 D). Hence, the lack of FXIII might be responsible for the more severe skin inflammation observed in FXIII-deficient mice. This was additionally verified by

the attenuation in the inflammatory reaction of *S. pyogenes*-infected FXIII-deficient mice after exogenous reconstitution with the human FXIII-concentrate Fibrogammin (Fg; Figure 4.20 E). These observations clearly indicated a protective role for FXIII during early host defense against *S. pyogenes* mediated skin infections.

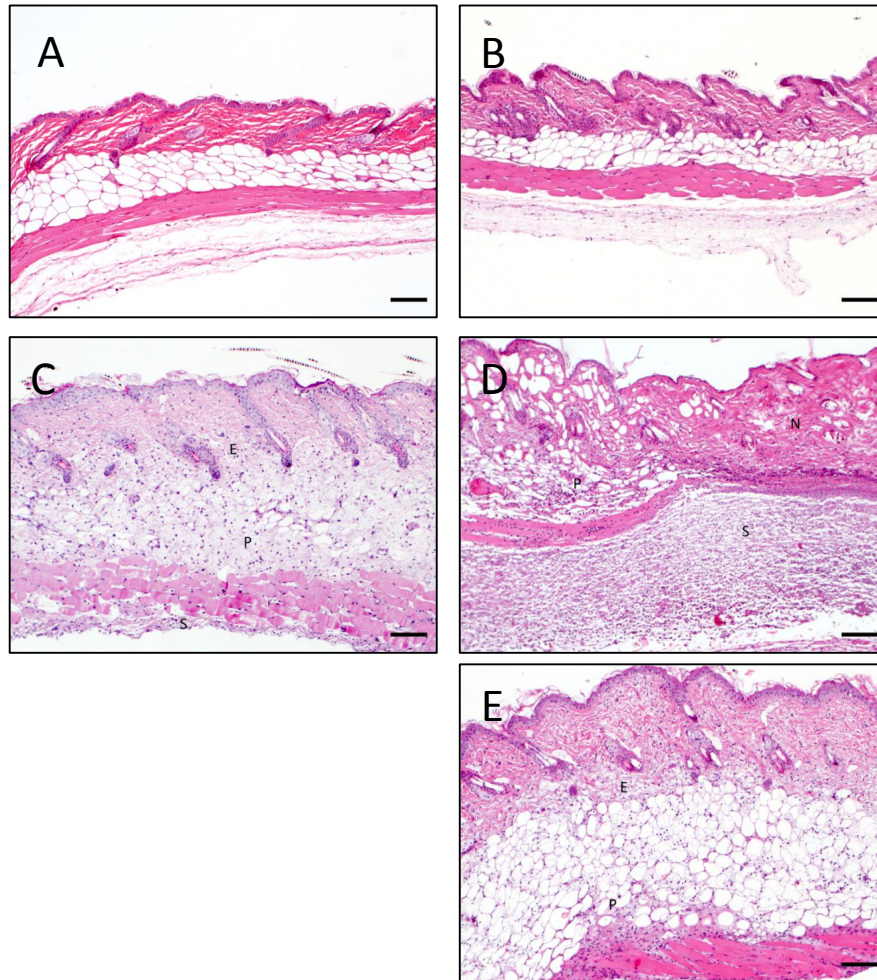


Figure 4.20: Histological examination of the local focus of *S. pyogenes* infection.

HE staining of representative skin sections from uninfected wild type (A) and FXIII-deficient mice (B) as well as *S. pyogenes*-infected wild type (C), FXIII-deficient (D) and Fibrogammin-reconstituted FXIII-deficient (E) mice at 24 h after bacterial inoculation. E = edema, P = panniculitis, S = subcutaneous inflammation, N = necrosis. All scale bars represent 100 μ m.

4.3.2 FXIII-deficient mice developed a more severe systemic response to *S. pyogenes* skin infection

As described above, an activation of FXIII was detected at the site of *S. pyogenes* infection 24 h after bacterial inoculation (Figure 4.18). Due to the important role of FXIII in the contact system, the activated partial thromboplastin time (aPTT), as measurement of contact system

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activation, was determined in the plasma of infected wild type and FXIII-deficient mice at progressive times after subcutaneous inoculation of *S. pyogenes* (Figure 4.21). As a control, the aPTT was measured in the plasma of uninfected wild type and FXIII-deficient mice ranging between 30 and 40 s (Figure 4.21). The aPTT increased up to 50 to 60 s in both wild type and FXIII-deficient mice at 24 h after *S. pyogenes* inoculation. This moderate increase in the aPTT indicates an activation of the contact system that might explain the local activation of FXIII, which was detected at the same time of infection. While the aPTT decreased at 48 h of infection in wild type mice and further remains at normal levels, the aPTT increased progressively in FXIII-deficient mice, reaching approximately 90 s at 72 h of infection (Figure 4.21). An extreme prolongation of the aPTT was previously described to indicate a systemic contact activation which is connected with the development of sepsis and severe infections (Herwald *et al.*, 2003; Oehmcke *et al.*, 2009). Moreover, a recent report has shown that the degree of prolongation of the aPTT correlates with the severity of *S. pyogenes* infections (Fiebig *et al.*, 2015). Therefore, the prolonged aPTT in infected FXIII-deficient mice might be an indication of a more severe systemic infection. However, the aPTT in FXIII-deficient mice treated with Fibrogammin was comparable to that of infected wild type mice (Figure 4.21) supporting a role for FXIII in the modulation of contact activation during *S. pyogenes* skin infection.

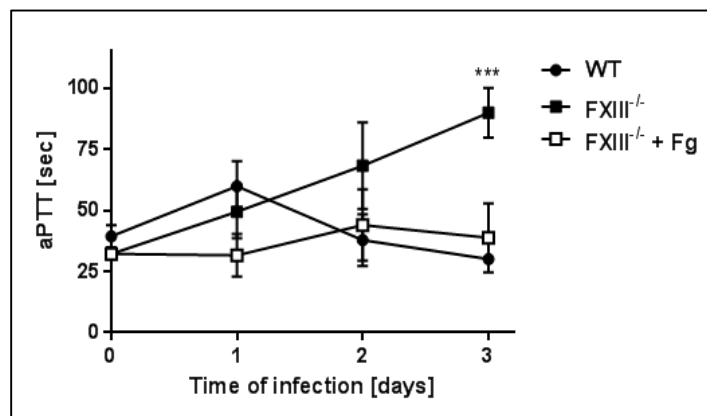


Figure 4.21: Activated partial thromboplastin time (aPTT) during *S. pyogenes* infection.

The aPTT was measured in the plasma of wild type mice (black circles) and FXIII-deficient mice (black squares) as well as FXIII-deficient mice which were reconstituted with Fibrogammin (white squares) after 24, 48 and 72 h of infection with *S. pyogenes*. Plasma obtained from uninfected wild type and FXIII-deficient mice served as control. The graph represents the mean \pm SD of 3 to 9 mice per group obtained from three independent experiments. Statistical significance was calculated by ANOVA test: *** $p < 0.001$.

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To further examine the impact of FXIII in host defense to *S. pyogenes* skin infection, the level of the pro-inflammatory cytokine interleukin-6 (IL-6) was determined in the serum of wild type and FXIII-deficient mice at progressive times after bacterial inoculation (Figure 4.22). For this purpose, wild type and FXIII-deficient mice were subcutaneously infected with *S. pyogenes* 5448 and the concentration of IL-6 was measured in the plasma obtained from infected mice by ELISA. While a moderated increase in the levels of IL-6 was measured in the plasma of all groups at 24 h of infection and remained at this moderated levels in wild type mice at 72 h of infection, a sharp increase in the level of the IL-6 level was observed in the plasma of FXIII-deficient mice at 72 h after bacterial inoculation (Figure 4.22). Interestingly, this increase in the plasma levels of IL-6 at 72 of infection was not observed after reconstitution of FXIII-deficient mice with Fibrogammin (Figure 4.22).

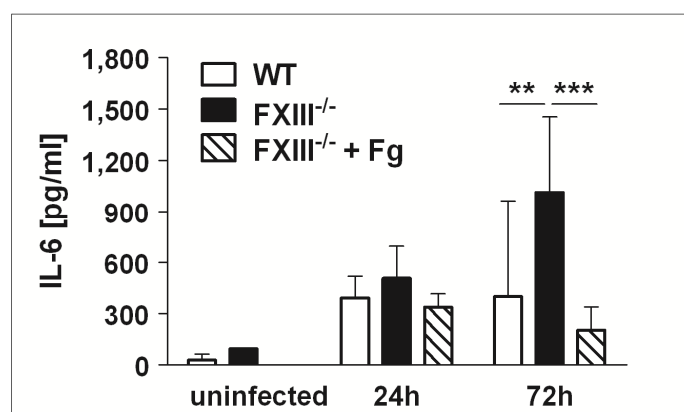


Figure 4.22: IL-6 levels in the plasma of *S. pyogenes*-infected mice.

Levels of IL-6 in the plasma of wild type (white bars), FXIII-deficient (black bars) and reconstituted FXIII-deficient (hatched bars) mice measured after 24 and 72 h of infection by ELISA. The graph represents the mean \pm SD of 3 to 9 mice per group obtained from three independent experiments. Statistical significance was calculated by ANOVA test: ** $p < 0.01$, *** $p < 0.001$.

Taken together, the results of the attenuated aPTT and moderate IL-6 production in the presence of FXIII (wild type and Fibrogammin-reconstituted FXIII-deficient mice) demonstrated a role for FXIII in the early innate immune response against *S. pyogenes* skin and soft tissue infections.

4.3.3 FXIII reduces the systemic dissemination of *S. pyogenes* from the local site of infection

To investigate a potential contribution of FXIII to limit bacterial spreading and systemic dissemination from the local site of infection at the skin, bacterial loads in blood, liver and spleen were determined in wild type and FXIII-deficient mice 72 h after subcutaneous

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inoculation of *S. pyogenes*. Significant higher amounts of bacteria were detected in blood (Figure 4.23 A) and spleens (Figure 4.23 B) of FXIII-deficient mice compared to wild type mice. Bacterial loads were also higher in livers of mice deficient in FXIII, but no significant differences were recorded in comparison to wild type animals (Figure 4.23 C). However, bacterial loads in blood and spleens of FXIII-deficient mice which were reconstituted with Fibrogammin were significantly reduced when compared to untreated FXIII-deficient animals (Figure 4.23). The reconstitution with Fibrogammin leads also to a decreased amount of bacteria in the livers, but did not reach statistic significance.

Taken together, these results indicate that FXIII contributed to the limitation of *S. pyogenes* systemic dissemination during skin infection. The FXIII-mediated entrapment of *S. pyogenes* at the site of infection may be the mechanism underlying this process.

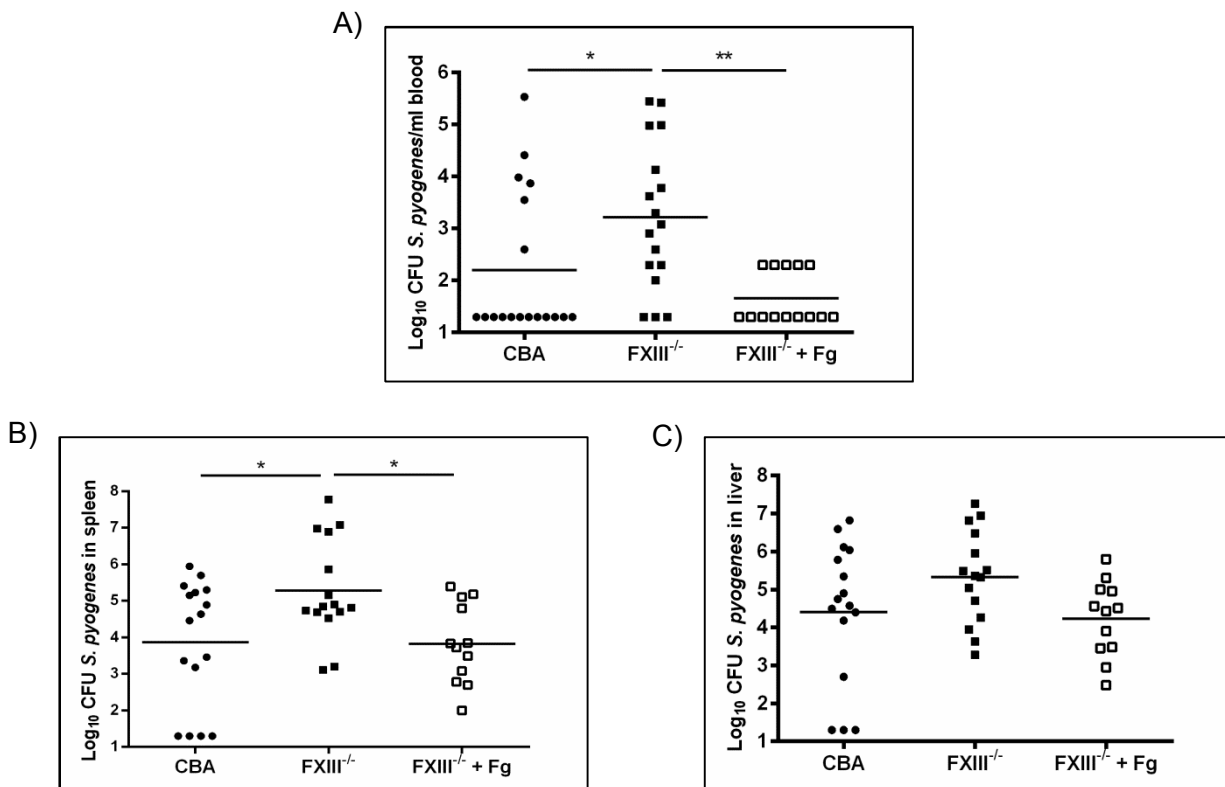


Figure 4.23: Bacterial loads in blood and systemic organs of *S. pyogenes*-infected mice at 72 h of infection.

Wild type (black circles), FXIII-deficient (black squares) and reconstituted FXIII-deficient mice (white squares) were infected with *S. pyogenes* 5448 and bacterial loads in blood (A), spleen (B) and liver (C) were determined after 72 h. The graphs represent the mean of 12 to 17 mice per group obtained from four independent experiments. Statistical significance was calculated by ANOVA test: * p < 0.05, ** p < 0.01.

4.3.4 FXIII improves the survival of mice during *S. pyogenes* skin infection

The beneficial role of FXIII during the early innate immune response against *S. pyogenes* skin infections was further investigated by recording the survival times of wild type, FXIII-deficient and Fibrogammin-reconstituted FXIII-deficient mice after subcutaneous infection with *S. pyogenes*. Whereas approximately 70% of infected wild type mice survived after 7 days of infection, 95% of FXIII-deficient mice succumbed to the infection (Figure 4.24 A). Reconstitution of FXIII-deficient mice with Fibrogammin improved the survival of these animals and only 10 % of the mice died by day 7 of infection (Figure 4.24 A). In addition, by day 6 of infection, the skin lesions of infected wild type mice were local and more superficial than the larger lesions associated with dermal necrosis and ulceration observed in FXIII-deficient mice (Figure 4.24 B). Fibrogammin-treated FXIII-deficient mice developed small lesions comparable to those of wild type mice (Figure 4.24 B).

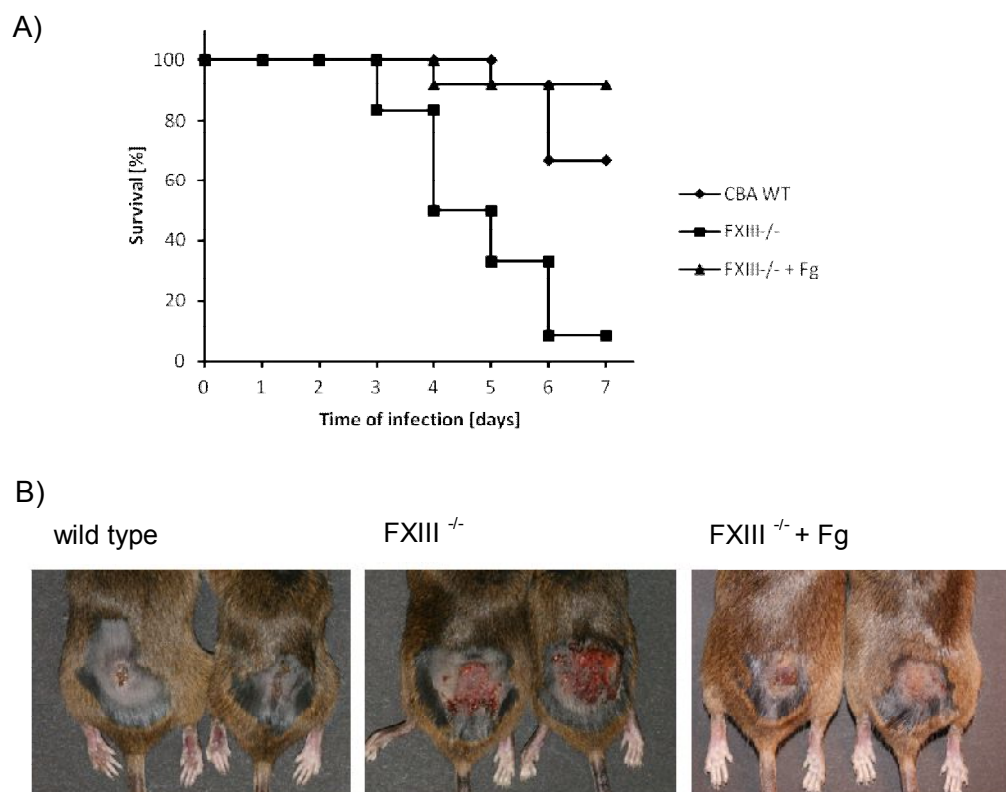


Figure 4.24: Influence of FXIII in mouse survival and skin lesion severity.

Infected wild type and FXIII-deficient mice as well as FXIII-deficient mice reconstituted with Fibrogammin were infected with *S. pyogenes* strain 5448 and the survival was monitored daily (A). Photographs show skin lesions of wild type (left panel), FXIII-deficient (middle panel) and Fibrogammin-reconstituted FXIII-deficient mice (right panel) at day 6 of infection (B). The survival curves shown in (A) represent data from 12 mice per group obtained from three independent experiments.

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In summary, the results obtained from the survival studies indicate an important role of FXIII during *S. pyogenes* mediated skin infections. Thus, FXIII limited the development of a systemic immune response and bacterial spreading resulting in an improved survival of infected mice.

5 DISCUSSION

During the last two decades, a growing body of evidence has been gathered demonstrating that coagulation is an integral part of the host innate immune response to pathogens (Delvaeye and Conway, 2009). Thus, contact activation was shown on the surface of gram-positive bacteria such as *S. pyogenes* and *S. aureus* as well as on the surface of gram-negative bacteria such as *E. coli* and *Salmonella*, leading to the induction of the entire clotting cascade including thrombin, fibrinogen and FXIII (Herwald *et al.*, 2003; Loof *et al.*, 2011b). Already in 2004, Sun and colleagues proclaimed a critical role of fibrin clot formation during *S. pyogenes* infections by using a subcutaneous murine infection model (Sun *et al.*, 2004). Further studies reported that the activation of the contact system could lead to the immobilization of pathogenic bacteria, including *S. pyogenes*, within the newly formed fibrin network. Thus, the bacterial entrapment was proposed as a new innate immune mechanism to prevent bacterial spreading from the site of infection (Shannon *et al.*, 2010; Wang *et al.*, 2010; Sun *et al.*, 2009; Loof *et al.*, 2011b). Coagulation FXIII was identified as a key factor that promotes the entrapment of pathogenic bacteria by cross-linking of bacterial surface structures with the fibrin network (Loof *et al.*, 2011b; Wang *et al.*, 2010). Furthermore, it was demonstrated that FXIII targets the M1 protein on the surface of *S. pyogenes* enabling bacterial entrapment (Loof *et al.*, 2011b). Previous studies postulated that the bacterial entrapment process is generally dependent of FXIII (Loof *et al.*, 2011b; Wang *et al.*, 2010). This hypothesis was supported by the observation that the gene encoding FXIII is highly conserved among the genome of vertebrates as well as invertebrates (Jiang and Doolittle, 2003). Accordingly, FXIII represents the only coagulation factor that has homologues in the coagulation cascades of all species studied so far (Jiang and Doolittle, 2003; Loof *et al.*, 2011a).

The main objective of this thesis was the investigation of the relevance of bacterial entrapment as an innate immune mechanism against streptococcal infections as well as the identification of key factors promoting this process. For this purpose, different bacterial species belonging to the genus *Streptococcus* were analyzed for their entrapment by *in vitro* clotting assays. The obtained experimental data demonstrated the efficient entrapment of *S. pyogenes* and SDSE. The entrapment of these bacteria was largely mediated by the expression of the M protein on the surface of *S. pyogenes* and of the M-like protein FOG on the surface of SDSE, as demonstrated by the reduced entrapment of these bacteria within fibrin networks when these surface proteins were lacking. In contrast to *S. pyogenes* and SDSE, no bacterial immobilization of *S. pneumoniae* and *S. oralis* was observed. These data

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lead to the suggestion that the entrapment of bacteria within fibrin clots does not represent a mechanism of host defense against streptococcal infections in general but it is rather restricted to a few species. *S. pyogenes* of serotype M1 was selected for further studies because this bacterium can be efficiently immobilized within fibrin clots and, more interesting, this immobilization is influenced and enhanced by FXIII. Thus, the immobilization of *S. pyogenes* M1 was significantly reduced in FXIII-deficient plasma clots. The dependency of FXIII for bacterial immobilization was limited to *S. pyogenes* of M1 serotype since other serotypes such as M1-2, M3, M6, M49 and M53 were also entrapped within the fibrin clot but this entrapment was not affected by the presence/absence of FXIII. Further experiments using different *S. pyogenes* strains of serotype M1 and their corresponding M1 deficient mutant strains verified previous observations and confirmed the influence of FXIII and the M1 protein on the entrapment of *S. pyogenes* M1 bacteria.

During the entrapment, *S. pyogenes* M1 bacteria become cross-linked within the fibrin network by the action of FXIII. The detailed mechanism and type of interaction between FXIII and the M1 protein of *S. pyogenes* has not been elucidated yet. Initially, a covalent linkage between FXIII and the M1 protein (Loof *et al.*, 2011b) was expected based on the enzymatic activity of FXIII as a transglutaminase catalyzing the formation of isopeptide bonds (Lorand and Graham, 2003). However, results obtained from experiments that were performed in the frame of this thesis rebutted a covalent linkage between FXIII and the M1 protein that seems to represent more a recognition structure targeted by FXIII for bacterial entrapment. It is already known for a long time that M proteins bind fibrinogen (Tillett and Garner, 1933). Fibrinogen binding is mediated by the B repeat region of the M protein located within the semi-variable domain (Ringdahl *et al.*, 2000). Fibrinogen also is the central factor of the coagulation cascade and represents the basic substance of the clot that ensures wound closure and prevents excessive blood loss after injury. Thrombin activates fibrinogen to fibrin that polymerizes into fibrin fibers. However, the formation of a stable fibrin network is mediated by FXIII-catalyzed cross-linking of fibrin molecules resulting in a stable clot.

To get further insights into the molecular mechanisms underlying the interactions of fibrinogen, FXIII and the M1 protein, *in vitro* fibrinogen conversion experiments were performed. The findings of these experiments showed that, in the absence of FXIII, recombinant streptococcal M1 protein inhibited the conversion of fibrinogen into fibrin, which is required for bacterial entrapment. This inhibitory effect was an exclusive feature of the M1 protein since it was not observed with recombinant M proteins of other serotypes or with the M-like protein FOG of SDSE. The inhibition of the fibrinogen conversion was also observed with whole *S. pyogenes* bacteria but only in case of serotype M1. Furthermore, deletion of the M1 protein in *S. pyogenes* resulted in loss of inhibition of fibrinogen conversion. These

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findings supporting the ability of M1 proteins to interfere with fibrinogen conversion in the absence of FXIII are in good agreement with the findings showing that FXIII exclusively mediated the entrapment of *S. pyogenes* M1 bacteria. Taken together, the possibility to inhibit fibrin formation might represent a new mechanism of *S. pyogenes* serotype M1 bacteria to prevent their entrapment by the coagulation system in the absence of FXIII.

To broaden the knowledge about the molecular mechanism behind the inhibition of the fibrin formation by the M1 protein, fibrinogen conversion experiments using streptococcal M1 protein constructs containing different M1 protein domains were performed. Interestingly, the A region of the M1 protein was identified to be responsible for the inhibition of the fibrinogen conversion. These results were confirmed by fibrinogen conversion experiments using heterologous expressed chimeric M1 and M3 proteins, whose A regions were exchanged, on the surface of *L. lactis*. Accordingly, the A1M3 protein, containing the A region of the M1 protein inhibited the conversion of fibrinogen in the absence of FXIII whereas the A3M1 protein which contains the A region of the M3 protein did not influence the fibrin formation. These experimental data supported the absolute requirement of the A region for inhibition of fibrinogen conversion by the M1 protein. Since the B repeats were described as the classical fibrinogen binding region of the M1 protein (Ringdahl *et al.*, 2000), the identification of the A region as the domain of the M1 protein responsible for the inhibition of fibrinogen conversion was surprising. However, since the A region is located in the hypervariable region of the M proteins that highly differs in sequence and structure among the different M serotypes (Facklam *et al.*, 1999), this could explain why this inhibitory mechanism is restricted to *S. pyogenes* of the M1 serotype.

Despite the identification of the A region as the domain of the M1 protein that inhibits fibrinogen conversion, the exact mechanism behind this interaction is still unknown. Nevertheless, binding to fibrinogen may be assumed to be a precondition for inhibition of fibrinogen conversion. In this regard, Macheboeuf and colleagues identified a new cryptic fibrinogen binding site within the A region of the M1 protein (Macheboeuf *et al.*, 2011). The capacity of the A region to bind fibrinogen was also demonstrated in this thesis by fibrinogen pulldown assays. Additionally, Macheboeuf *et al.* demonstrated that fibrinogen and the M1 protein form a pathological network capable to activate neutrophils to release of critical amounts of the potent vasodilator heparin-binding protein (HBP) (Macheboeuf *et al.*, 2011), which causes vascular leakage and tissue injury during STSS (Linder *et al.*, 2009). As the inhibition of fibrinogen conversion by the M1 protein was not observed in the presence of FXIII, it can be speculated that FXIII might contribute to host defense by supporting bacterial entrapment after interfering with the capacity of *S. pyogenes* M1 to inhibit clot formation.

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However, these considerations do not explain the mechanism behind the inhibition of fibrinogen conversion by the A region of the M1 protein. A potential inhibition of thrombin, the enzyme catalyzing the conversion of fibrinogen into fibrin, was discarded after demonstrating that thrombin was active in fibrinogen conversion experiments in the presence of the M1 protein by using the chromogenic substrate S-2238 (data not shown). An alternative mechanism could be that the binding of fibrinogen to the A region of the M1 protein might lead to a conformational change within the fibrinogen molecule resulting in the inaccessibility of the thrombin cleavage sites on the fibrinogen molecule. Thrombin cleaves the fibrinopeptides A and B (FPA and FPB) from the fibrinogen α - and β -chains at arginine residues 16 and 14, respectively (Binnie and Lord, 1993). After cleavage of FPA and FPB, fibrin monomers polymerize to form fibrin fibers and networks. Further research addressing the structural analysis of fibrinogen bound to the A region in comparison to fibrinogen bound to the B repeat region of the M1 protein might help to clarify this hypothesis.

Furthermore, the relevance of this inhibitory mechanism for the pathogenesis of *S. pyogenes* remains unclear, especially by considering the fact that this mechanism seems to be restricted to the serotype M1. This serotype is one of the most frequently appearing *S. pyogenes* strains worldwide causing invasive diseases (Steer *et al.*, 2009). It can be speculated that the capacity of this bacteria to interfere with the clot formation and, thus, with the bacterial entrapment could contribute to the pathogenesis of this successful serotype. Binding of fibrinogen to the M protein to avoid the recognition of the bacteria by the host immune system has been proposed as an important strategy of *S. pyogenes* to resist phagocytosis (Whitnack *et al.*, 1983; Horstmann *et al.*, 1992). The potential contribution of the inhibition of fibrinogen conversion as mechanism to preserve intact fibrinogen on the bacterial surface and, thus, to resist phagocytosis by PMNs was also investigated. As the inhibition of fibrinogen conversion is a property of the M1 protein, the resistance to phagocytosis of *L. lactis* expressing M1 protein was compared with that of *L. lactis* expressing the M1 protein where the A region has been exchanged for that of the M3 protein (A3M1). However, no significant differences were observed in the resistance to phagocytosis of these two *L. lactis* strains (data not shown).

In conclusion, the inhibition of the fibrinogen conversion by the M1 protein of *S. pyogenes* could prevent bacterial entrapment within a fibrin network at the site of infection and could promote the capacity of *S. pyogenes* to disseminate from local site of infection. Therefore, this mechanism could be used by *S. pyogenes* M1 for immunoevasion. However, this mechanism is only active in the absence of FXIII. This coagulation factor interferes with the inhibition of fibrinogen conversion and promotes the immobilization of *S. pyogenes* M1 within the fibrin clot. Thus, FXIII contributes to the early host defense against this pathogen. This

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was previously demonstrated by Loof and colleagues (Loof *et al.*, 2011b) by showing that mice lacking FXIII exhibited more severe signs of inflammation at the site of *S. pyogenes* infection when compared to wild type mice. A deeper investigation of the role played by FXIII in the host defense against *S. pyogenes* M1 skin infection was performed also in the frame of this thesis.

After *S. pyogenes* invasion of the host, both the contact system and the innate immune components such as macrophages, DCs and PMNs are rapidly activated. Though coagulation is assumed to play a role during the very early phase of local infections, fibrin network formation and pathogen entrapment help to restrict the pathogen at the site of infection and to enable their fast elimination by the innate immune system. Thus, coagulation and especially the FXIII-mediated immobilization seems to play a major role during local skin and soft tissue infections caused by *S. pyogenes* of serotype M1. This hypothesis was supported by *in vivo* studies reporting that, after intravenous or intraperitoneal *S. pyogenes* administration, fibrin clot formation could not prevent the dissemination of *S. pyogenes* and the establishment of an infection (Sun *et al.*, 2004; Shannon *et al.*, 2010). In experiments performed in the frame of this thesis, wild type and FXIII-deficient mice were subcutaneously infected with the *S. pyogenes* strain 5448 of serotype M1T1. This globally disseminated subclone of the serotype M1T1 has been linked to the resurgence of severe invasive *S. pyogenes* infections observed since the 1980s in the western world and causes severe skin and soft tissue infections (Aziz and Kotb, 2008). Experimental data demonstrated that mice deficient in FXIII were much more susceptible to *S. pyogenes* M1-mediated skin infection than wild type mice as shown by the shorter survival times, the higher levels of bacterial dissemination from the local site of infection and the more intense systemic inflammation. In particular, FXIII-deficient mice exhibited higher levels of IL-6 at later times of infection and prolonged aPTT than wild type mice. Prolongation of aPTT is an indicator of contact activation that was also detected on the surface of *S. pyogenes in vitro* subsequently leading to the induction of the entire coagulation cascade (Loof *et al.*, 2011b). Contact activation *in vivo* was also detected in human and mouse plasma after *S. pyogenes* infection (Sriskandan *et al.*, 2000) and an extreme prolongation of this clotting parameter was observed during severe *S. pyogenes* infections (Herwald *et al.*, 1998; Oehmcke *et al.*, 2009). Indeed, abnormal systemic contact activation can lead to an overwhelming inflammation characterized by the release of massive amounts of vasoactive kinins such as BK that can contribute to the development of the most severe clinical outcomes of *S. pyogenes* infections such as sepsis and STSS (Oehmcke *et al.*, 2009; Pixley *et al.*, 1995; Henningham *et al.*, 2012). Thus, a careful regulation of coagulation is necessary during the innate immune response against *S. pyogenes* infection to maintain hemostasis under physiological levels.

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The higher bacterial loads in blood and organs detected in mice deficient in FXIII when compared to wild type mice after 72 h of *S. pyogenes* skin inoculation support the concept that FXIII-mediated entrapment at the site of infection might prevent systemic dissemination of bacteria. As a proof of principle, FXIII-deficient mice reconstituted with Fibrogammin, a human FXIII concentrate that was subcutaneously administered after *S. pyogenes* infection, exhibited a reduced bacterial spreading in comparison to wild type mice and only moderate inflammation indicated by just slightly increased aPTT and IL-6 levels. Furthermore, FXIII-reconstitution also improved the survival of FXIII-deficient mice after *S. pyogenes* infection to a rate that was even higher than the survival rates of wild type mice.

Besides retention of bacteria at the site of infection to prevent their spreading by host coagulation, it has further been shown that bacteria are eliminated within the fibrin clot by antimicrobial activity. Thus, AMPs derived from components of the coagulation cascade, such as fibrinogen, thrombin and HK might promote the killing of entrapped bacteria (Påhlman *et al.*, 2013; Frick *et al.*, 2006; Papareddy *et al.*, 2010; Nordahl *et al.*, 2005). Furthermore, the killing of *S. pyogenes* and other fibrinogen-binding bacterial pathogens within thrombin activated human plasma was demonstrated (Loof *et al.*, 2011b, Påhlman *et al.*, 2013). These data suggest that within a fibrin clot AMPs reach effective concentrations to kill entrapped bacteria.

In conclusion, the results of this thesis confirmed that FXIII exerts an important function in the early innate immune defense against skin infections caused by *S. pyogenes* bacteria of serotype M1. Upon coagulation activation in response to infection, activated FXIII was shown to target the M1 protein, which leads to the immobilization of bacteria within the fibrin network. In the absence of FXIII, the entrapment of *S. pyogenes* M1 bacteria is significantly reduced due to the inhibition of the conversion of fibrinogen to fibrin mediated by the M1 protein. This ability might represent a novel virulence mechanism of *S. pyogenes* to avoid their entrapment and to promote bacterial dissemination in places where FXIII is absent.

6 FUTURE OUTLOOK

The results of this thesis confirmed the protective role of FXIII during the early host defense against *S. pyogenes* of serotype M1 and support the concept of a potential therapeutic use of FXIII. However, the therapeutic potential of FXIII during *S. pyogenes* mediated infectious diseases needs to be further investigated. Since *S. pyogenes* bacteria have been shown to induce fibrinolysis by the secretion of streptokinase (SK) (Tillett and Garner, 1933; Green, 1948), this pathogen might be able to use SK to escape from a fibrin clot. SK is a highly specific protein that activates human plasminogen to plasmin, the main protease of the fibrinolytic system (Danø *et al.*, 1985; Marcum and Kline, 1983; Walker *et al.*, 2005). Because SK is specific for human plasminogen and does not activate murine plasminogen, studies addressing the role of SK need to be performed with transgenic mice expressing human plasminogen (hPLG). These mice have been shown to be more susceptible to subcutaneously inoculated *S. pyogenes* bacteria when compared to wild type mice (Sun *et al.*, 2004). The increased susceptibility is thought to be caused by the ability of *S. pyogenes* to induce fibrinolysis leading to an increased bacterial dissemination from the site of infection. However, an impact of FXIII during these interactions has not been demonstrated yet. Thus, infection experiments in which hPLG mice will be treated with Fibrogammin after inoculation of *S. pyogenes* might be a possibility to further investigate if FXIII could counteract or reduce SK-mediated fibrinolysis. However, SK was already identified as a target for the treatment of severe *S. pyogenes* infections and drugs that inhibit the production of SK have been shown to improve the survival of mice after bacterial inoculation (McArthur *et al.*, 2012; Sun *et al.*, 2012). Thus, a combination of both, the blockage of SK mediated fibrinolysis and the local increase of FXIII activity at the site of infection might be a concept for a more efficient treatment of *S. pyogenes* skin and soft tissue infections.

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ACKNOWLEDGEMENT

I would like to express my gratitude to all those who have contributed to the success of this PhD thesis:

First of all, I thank **Prof. Dr. Eva Medina** for the warm welcome in her research group, the continuous interest in the progress of my PhD project and her support during the preparation of manuscripts as well as this PhD thesis.

I thank **Prof. Dr. Gursharan Singh Chhatwal** for the opportunity to perform the laboratory work of this thesis in the department of medical microbiology.

I would like to sincerely thank my supervisor **Dr. Torsten Loof** who had the idea for this project and gave me the chance to work on it. Thank you for your support during your time at the HZI and during the time you already left the HZI.

Furthermore, sincere thanks go to **Prof. Dr. Manfred Rohde** as the mentor of my PhD thesis and as a member of my thesis committee. In addition to that I thank to whole ZEIM group for their excellent electron microscopic analyzes.

I am grateful to **Prof. Dr. Susanne Engelmann** for the support of this thesis as the second referee, **Prof. Dr. Ralf Schnabel** for his agreement to be the chair in my disputation as well as **Prof. Dr. Maren von Köckritz-Blickwede** for her interest in my PhD project as a member of my thesis committee.

I thank **PD Dr. Daniel Patric Nitsche-Schmitz** and **Dr. Giuseppe Gulotta** for providing some of the recombinant M protein constructs, their interest and support of this project of my PhD thesis.

I would also like to thank Gerhard Dickneite from CSL Behring for providing Fibrogammin®P and FXIII-deficient mice used in this study.

Furthermore, I thank Dr. Marina Pils and Anna Rinkel (Mouse Histology, HZI) for the excellent histopathological analyzes as well as Dr. Linda Johansson and Bhavya Chakrakodi from the Center for Infectious Medicine, Department of Medicine, Karolinska Institute, Karolinska University Hospital Huddinge (Stockholm, Sweden) for excellent double immunofluorescence analysis.

I will also take the chance to acknowledge the President's Initiative and Networking Fund of the Helmholtz Association of German Research Centers and the HZI graduate school for the

ACKNOWLEDGEMENT

funding of this PhD thesis and the opportunity to participate in laboratory and scientific courses as well as conferences.

I am so much obliged to all the members of the former **department of medical microbiology** and the **research group infection immunology** who supported me during the laboratory work, the breaks and the time of writing this thesis. I had a wonderful time with all of you and I will miss you a lot!

Special thanks also go also to **Dr. René Bergmann, Dr. Oliver Goldmann and Dr. Andreas Itzek** for their interest, critical and constructive discussions and their helpful advices!

Furthermore, I am very grateful to my fellow PhD students and friends **Katharina Rox, Anja Ochel, Mathias Scheb-Wetzel, Katja Branitzki-Heinemann** as well as **Anja Eisenmann, Robert Thänert and Guiseppe Gulotta** for the pleasant atmosphere during the happy and less happy times in the life of a PhD student!

I still acknowledge **Caroline Wesener** for her excellent work during her Master thesis. Your hard work on the chimeric M proteins and the corresponding *Lactococcus* mutants helped me a lot on my way.

I will not forget **my friends outside the “scientific world”** which also greatly contributed to the success of this PhD thesis. You all helped me through difficult times with motivation, interest and wonderful distraction. At this point I will especially mention **Wiebke** for carefully reading parts of the manuscript of this thesis and **Ulli** for the help with the german abstract.

Finally, I am so much obliged to my whole family, especially my **parents** and my brother **Sebastian**! Your imperturbable faith in me gave me the confidence to follow my interests! You never doubt about me and you always supported me on the way I had gone so far. At least, but most importantly, I thank you, **René**, for your love, your care and your support all the years that required a lot of concessions and comprises. I hope we will go forward on our way together!